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THE HUMAN SOLUTE CARRIER 26 FAMILY OF ANION EXCHANGERS

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Academic dissertation

To be publicly discussed with the permission of the Faculty of Medicine, University of Helsinki, in the Small Lecture Hall at Haartman Institute, Haatmaninkatu 3, Helsinki, on the 11th of September at 12 noon.

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To my wife and children

CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on four original publications that are referred to by their Roman numerals in the text. In addition some unpublished data are presented.

I. Lohi H., Kujala M., Kerkelä, E., Saarialho-Kere, U., Kestilä M. and Kere J. Mapping of five new putative anion transporter genes in human and characterization of SLC26A6, a candidate gene for pancreatic anion exchanger. Genomics 70:102-112, 2000.

II. Lohi H., Kujala M., Mäkelä S., Lehtonen E, Kestilä M., Saarialho-Kere U., Markovich D. and Kere J. Functional characterization of three novel tissue-specific anion exchangers: SLC26A7-A9. J. Biol. Chem. 277:14246-14254, 2002.

III. Lohi H., Lamprecht, G., Markovich D., Heil A., Kujala M., Seidler U. and Kere J. Isoforms of putative anion exchanger SLC26A6 (PAT1) mediate sulfate and chloride transport and have functional PDZ interaction domains. Am. J. Physiol. Cell Physiol., revised version submitted, 2002.

IV. Lohi H.*, Mäkelä S.*, Pulkkinen K. Höglund P., Karjalainen-Lindsberg M-L., Puolakkainen P. and Kere J. Upregulation of CFTR expression but not SLC26A3 and SLC9A3 in ulcerative colitis. Am. J. Physiol. 283: G567-575, 2002.
* equally contributed

ABBREVIATIONS

ACG1B	achondrogenesis type 1B
AE	anion exchanger
AO2	atelosteogenesis type 2
cAMP	cyclic adenosine 3´5´-monophosphate
CF	cystic fibrosis
CFEX	chloride-formate exchanger
CFTR	cystis fibrosis transmembrane regulator
CLD	congenital chloride diarrhoea
DIDS	4,4´-diisothiocyano-2,2´-disulfonic acid stilbene
DRA	down-regulated in adenoma
dRTA	distal renal tubular acidosis
DTDST	diastrophic dysplasia sulfate transporter
EBP50	ezrin-binding phosphoprotein 50
E3KARP	Na^+/H^+ exchanger 3 kinase A regulatory protein
ENaC	electrogenic sodium channel
EST	expressed sequence tagged site
G3PDH	glyceraldehydes-3-phosphodehydrogenase
HEVEC	high endothelial venule cell
htgs	high-throughput genomic sequence
HUGO	Human Genome Organization
NBC	sodium bicarbonate cotransporter
NCBE	sodium dependent chloride bicarbonate exchanger
NDAE1	Na-driven Cl^-/base exchanger 1
NHE3	sodium/hydrogen exchanger 3
NHERF	sodium hydrogen exchanger regulator factor
OMIM	online Mendelian Inheritance in Man
PBC	primary biliary chirrhosis
PDS	Pendred syndrome
PDZ	PSD-95/Disc-large/ZO-1 domain
RACE	rapid amplification of cDNA ends
rMED	recessive multiple epiphyseal dysplasia
SAO	Southeast Asian ovalocytosis
sat-1	rat sulfate anion transporter1
SLC	solute carrier
STAS	sulfate transporter and anti-sigma factor antagonist domain
Tat1	testis anion transporter 1

SUMMARY

Anion exchangers play a key role in the maintenance of many physiological processes in the body by transporting different anions such as Cl^-, HCO_3^-, SO_4^{2-} and oxalate across cell membranes. They participate in the CO_2 metabolism, contribute to the regulation of the cell growth, shape, death, metabolism and contractility, and regulate intracellular pH, cell volume as well as ion gradients. Two structurally different groups of anion exchangers have been found in human. A classical anion exchanger (AE) gene family consists of at least three members, which facilitate a Na^+-independent electroneutral exchange of Cl^- for HCO_3^- across the plasma membranes of mammalian cells. AE1 (band 3), which has been recently renamed as solute carrier family 4A member 1, SLC4A1, was the first anion exchanger identified, followed by its homologs, SLC4A2 (AE2) and SLC4A3 (AE3). The AE anion exchangers form a subfamily of the larger SLC4 family of bicarbonate and anion transporters.

We have focused our attention on the clearly distinguishable group of the previously so-called sulfate transporters, recently redefined functionally as the second anion exchanger gene family SLC26. Interestingly, the first three human genes, SLC26A2, SLC26A3 and SLC26A4 associate with phenotypically different disorders, involving cartilage growth, gut absorption, and hearing, respectively. SLC26 family is well conserved across taxonomic span, and our observation that the first fully sequenced animal, *C. elegans*, has seven putative members of this gene family prompted us to hypothesize that many more than three genes with important physiological functions or disease associations might exist also in human, stimulating the current study. By a genome-driven approach, we identified and mapped seven novel loci (designated SLC26A1, A6-A11) with distinct tissue expression patterns. Subsequently, we have systematically characterized the structure, expression and function of the SLC26A6-A9 genes using different molecular biological and physiological protocols.

The first new member, named SLC26A6, maps to chromosome 3p21.3 and encodes a predicted 738 amino acid transmembrane protein. The SLC26A6 protein is structurally highly homologous to the previous tissue-specific anion transporters. It is expressed at highest levels in the kidney and pancreas, and more specifically, in a subgroup of tubular cells in the kidney and in two pancreatic ductal cell lines. We identified also two alternatively spliced variants of SLC26A6, named SLC26A6c and SLC26A6d with tissue-specific expression patterns. Functional expression of the three variants in *Xenopus* oocytes demonstrated DIDS- and HCO_3^- -sensitive chloride and sulfate transport activity. Furthermore, we demonstrated that the C-terminus of SLC26A6 binds to the first

and second PDZ domains of the E3KARP and NHERF proteins in vitro. Truncation of the last three amino acids (TRL) of SLC26A6 abrogated the interaction, but did not affect transport function. These results demonstrate that SLC26A6 and its two splice variants can function as anion transporters linked to PDZ-interaction pathways. Our results support the general concept of microdomain organization for ion transport, and suggest a mechanism for CFTR-mediated SLC26A6 upregulation in pancreatic duct cells.

In addition, three novel members, named SLC26A7, SLC26A8 and SLC26A9, were identified on chromosomes 8, 6 and 1, respectively. The SLC26A7-A9 genes show tissue-specific expression in kidney, testis and lung, respectively. Localisation by immunohistochemistry and/or in situ hybridization localized SLC26A7 to distal segments of nefrons, SLC26A8 to developing spermatocytes, and SLC26A9 to the lumenal side of the bronchiolar and alveolar epithelium of lung. Expression of SLC26A7-A9 proteins in *Xenopus* oocytes demonstrated chloride, sulfate and oxalate transport activity, suggesting that they encode functional anion exchangers. The novel genes are likely to participate in important physiological functions involving anion transport in different organs and might associate with disorders.

In this study, we have also compared the expression of three major mediators of the intestinal salt transport, SLC26A3 (Cl^-/HCO_3^--exchanger), SLC9A3 (Na^+/H^+-exchanger) and CFTR (Cl^--channel), between healthy and inflamed colonic mucosa to understand the pathophysiology of diarrhoea in inflammatory bowel disease. All three transporters do likely play in concert with each other, and their functional defects have been associated with diarrhoeal diseases. The activity of inflammation was determined by histological evaluation of the peroperative colonic samples graded in three subgroups (mild, moderate and severe), and the expression levels were measured by real-time quantitative RT-PCR, immunohistochemistry and immunoblotting. Our results revealed the upregulation of CFTR expression but not SLC26A3 and SLC9A3 in ulcerative colitis, suggesting that the inhibition of CFTR might contribute to the therapy of diarrhoea in UC in the future.

1. INTRODUCTION

A relatively constant cellular homeostasis is essential for body functions. The cytoplasmic pH must be maintained within a narrow range in cells, while generating and responding to metabolic acid and base loads, and while maintaining acidic or alkaline environments within distinct subcellular compartments. To maintain the cell polarity and other functions, different concentrations of electrolytes in intra- and extracellular spaces must be tightly controlled by specific ion transport systems. Inorganic anions such as Cl^-, HCO_3^- and SO_4^{2-}, comprise an important group of electrolytes in body fluids. Chloride is the most abundant anion in plasma, and it has many physiological roles in maintaining the cell polarity, cell volume, water absorption, and in transporting other ions such as sodium, bicarbonate and protons. Bicarbonate along with CO_2 comprises the major pH buffer of biological fluids, and it enhances the solubility of proteins and ions in biological fluids. Sulfate is an important constituent of macromolecules such as mucins and proteoglycans, and the regulation of the sulfate transport is required for proper cell growth and development of the organism.

Hydrophilic, water-soluble anions cannot pass freely through membranes and need specific transport systems. Biochemical and biophysical studies on the red blood cell membrane proteins have paved the way for the identification of the mammalian anion transport systems (Kopito and Lodish 1985). The most abundant protein of the red cells, Band 3 (AE1, SLC4A1), was the first identified anion exchanger, exhibiting a Na^+-independent Cl^-/HCO_3^- exchange activity. Subsequently, several additional SLC4A1-related genes have been cloned (Kopito 1990). More recently, the second structurally different anion exchanger family, SLC26, has been established mainly by positional cloning of disease genes (Kere et al. 1999).

Anion exchangers have fundamental roles in many physiological processes such as maintenance of cell volume and intracellular pH. Intracellular pH is regulated through coordinated action of several acid-base transporters (Fig. 1), including Na^+/H^+ exchangers (NHEs), Na^+/HCO_3^- cotrasporters (NBCs), Na^+-dependent Cl^-/HCO_3^- exchangers (NCBEs), and Na^+-independent Cl^-/HCO_3^- anion exchangers (AEs) (Puceat et al. 1995; Soleimani and Burnham 2001). Intracellular pH is a major regulator of diverse cellular processes including metabolic pathways, Ca^{2+} homeostasis, cell contractility, cell excitability, gene expression and cell death (Fabiato and Fabiato 1978; Fry and Poole-Wilson 1981; Busa and Nuccitelli 1984; Orchard and Kentish 1990; Isfort et al. 1993; Kusuoka et al. 1993; Gottlieb et al. 1996; McConkey and Orrenius 1996). Disruption of anion

metabolism leads to acid/base disturbances as well as other electrolyte abnormalities that may predispose to different pathophysiological conditions.

Importantly, defects in the human anion transport systems have been associated with a wide range of distinct genetic diseases including spherocytic anemia, distal renal tubular acidosis, skeletal dysplasias, chloride diarrhoea, and congenital hearing loss with variably penetrant goiter (Hästbacka et al. 1994; Höglund et al. 1996; Everett et al. 1997; Alper 2002). Anion transport is also impaired in other pathophysiological states such as intestinal inflammations. By exploring the pathogenesis of these abnormalities much can be learned about the anion transport physiology in different parts of the body.

An alternative route for expanding our understanding of the transport physiology can benefit from the tremendous advances that have been made in human genetics in recent years. The fruits of the genome projects are facilitating the exploration of gene families and the identification of genes underlying different pathological conditions. An ever growing number of genomic sequences from different species is rapidly accumulating in databases allowing feasible identification, mapping and cloning of new genes. As a result, while new transport systems will emerge, many already functionally recognized transporters are also likely to get their molecular identity.

As an example of the fruits of genome projects, the current study describes the expansion of the SLC26 family using a genome-driven approach. Seven new putative anion exchangers were identified in human. They are likely to possess important physiological functions or disease associations, providing new insights to anion transport physiology in different parts of the body.

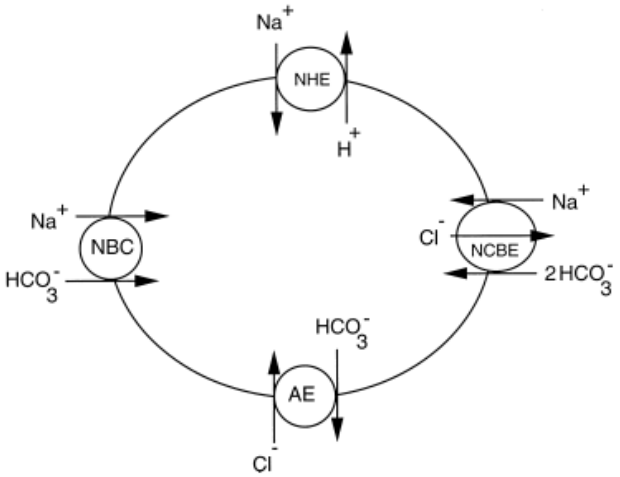

Figure 1. A schematic representation of acid-base transporters (adapted from Soleimani and Burnham 2001).

2. REVIEW OF THE LITERATURE

2.1. The AE anion exchangers belongs to the SLC4 bicarbonate superfamily

A classical family of at least three structurally and functionally related anion exchanger (AE) genes, AE1-AE3, has been identified from different species (Alper 1994). These anion exchangers constitute a diverse family of transporters, which contribute to the maintenance of transmembrane anion gradients, cell volume, and acid-base homeostasis.

Table 1. Nomenclature of the SLC4 gene family. SLC4A6 has been withdrawn because of redundancy with SLC4A7 and SLC4A8.

Symbol	Alternative name	Chromosomal location	OMIM Number	GenBank Accession
SLC4A1	AE1	17q12-q21	109270	XM008364
SLC4A2	AE2	7q35-q36	109280	XM032121
SLC4A3	AE3	2q36	106195	XM002605
SLC4A4	NBC1	4q21	603351	AF007216
SLC4A5	NBC4	2p13	-	AF207661
SLC4A7	NBC2	3p22	603353	AB012130
SLC4A8	NBC3	Chr 12	605024	AF069512
SLC4A9	AE4	Chr 5	-	AF332961
SLC4A10	NBCE	2q23-q24	605556	AB033759

The AE family comprises a subfamily within the larger SLC4 bicarbonate transporter superfamily (Fig. 2) that includes also several Na^+/HCO_3^- cotransporters (NBC) and two NBC-related genes, AE4 (NBC5, SLC4A9) and NDAE1 (Na-driven Cl^-/base exchanger 1) (Soleimani and Burnham 2001). Of interest, AE4 elicits Na^+-independent Cl^-/HCO_3^- activities, but shows higher structural homology to the members of the NBC family, being a structure intermediate between the NBCs and SLC4 proteins (Tsuganezawa et al. 2001). AE4 has been detected only in the apical membranes of HCO_3^--secreting β-intercalated cells in kidney. Since the HUGO Nomenclature Committee has recently revised the nomenclature of the gene family, the new SLC4A1-A3 symbols (AE1-AE3, respectively) will be used throughout this review (Table 1).

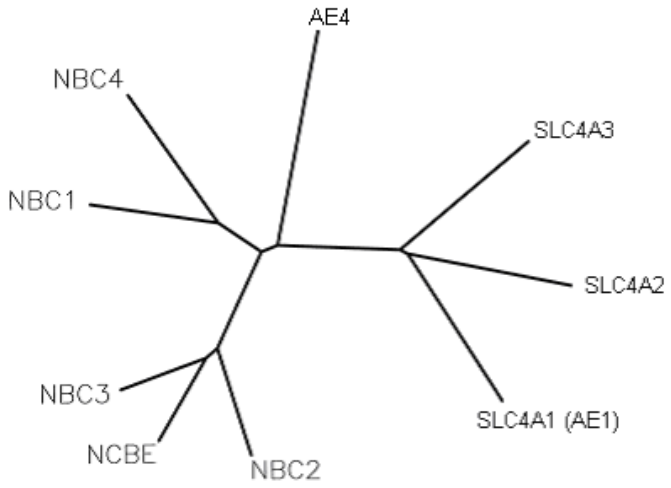

Figure 2. The evolutionary relationships among the mammalian SLC4 family of bicarbonate and anion transporters.

2.1.1. Characteristics of SLC4A1-A3

SLC4A1 is the most abundant protein of the red blood cell membrane, representing 10% of the surface area and ~25% of the total protein of the purified membrane (Steck et al. 1976). Because SLC4A1 is easily available, it has become one of the most studied membrane proteins. Although its functional and structural properties have been under extensive studies for many decades, the characterization of the primary structure had to await the isolation of the SLC4A1 cDNA clones (Kopito and Lodish 1985; Cox and Lazarides 1988; Tanner et al. 1988). Previous studies by direct peptide sequencing had yielded only a portion of the N-terminal domain and fragments from the membrane domain (Mawby and Findlay 1982; Brock et al. 1983; Kaul et al. 1983). To date, the SLC4A1 gene has been cloned from several different species (Alper 1994).

The human SLC4A1 gene maps to 17q21 (Showe et al. 1987) and encompasses 20 kb of DNA consisting of 20 exons. The cDNA sequence of 4906 nucleotides encodes a 911-amino-acid transmembrane glycoprotein (~100 kDa), with two domains having independent functions (Schofield et al. 1994). The amino-terminal 40-kDa of the SLC4A1 protein is located in cytoplasm and interacts with different cytoskeletal proteins, while the 55-kDa C-terminal membrane domain carries out the exchange of anions (Low 1986; Tanner 1997). SLC4A1 is glycosylated with a single

N-glycan chain at Asn[642], which is not needed for the transport function (Jay 1986; Casey et al. 1992).

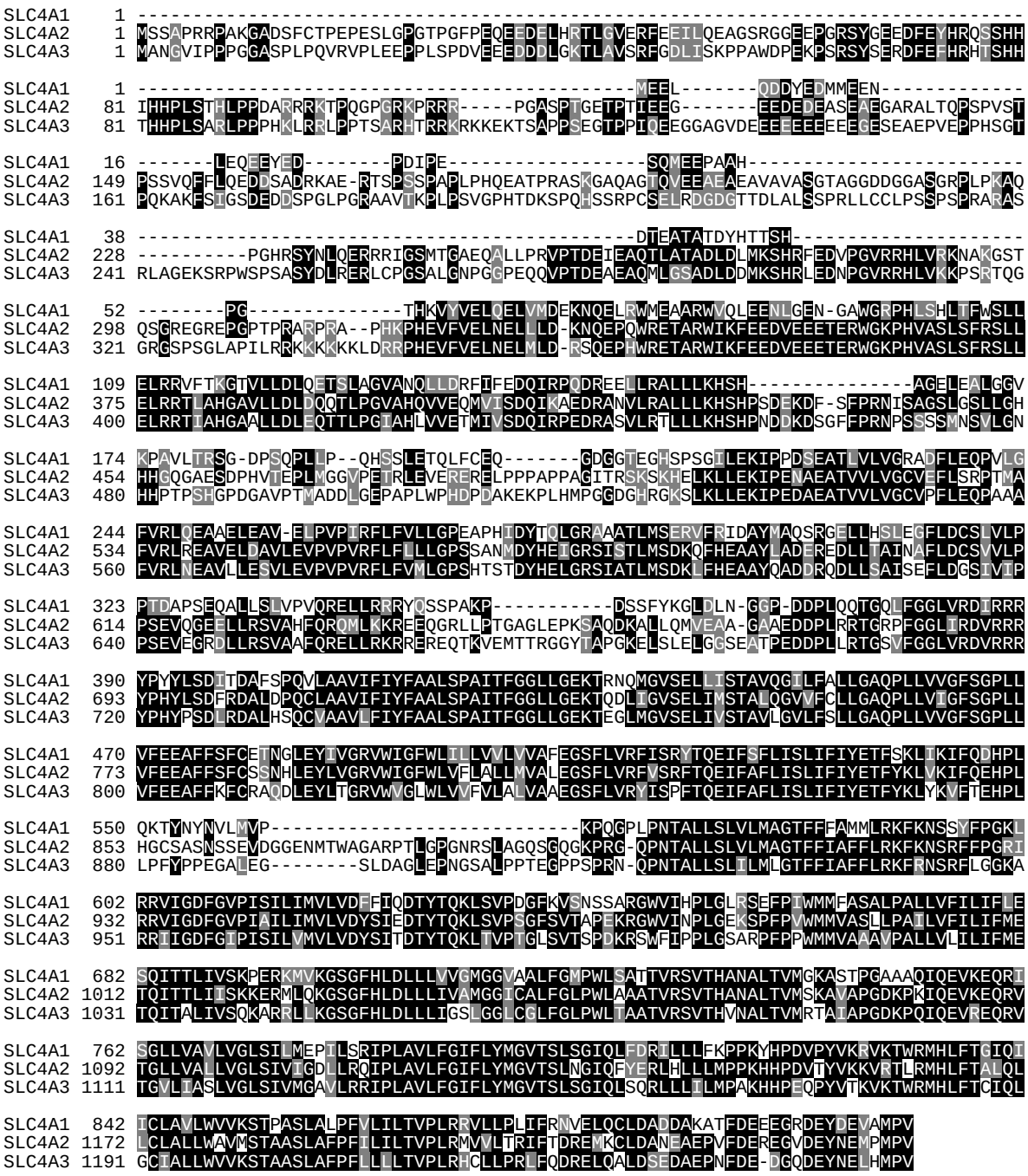

Figure 3. Multiple alignment of the anion exchangers of the SLC4 family. The identical and conserved residues are shown in black and gray, respectively.

Soon after the identification of SLC4A1, two related genes, SLC4A2 and A3, were cloned from numerous species by low stringency hybridizations (Demuth et al. 1986; Alper et al. 1988; Kopito et al. 1989; Kudrycki et al. 1990; Lindsey et al. 1990; Gehrig et al. 1992). The SLC4A2 gene maps to 7q35-q36 (Palumbo et al. 1986) and extends over 17 kb of genomic sequence. The cDNA

consists of 23 exons and 22 introns. Nine exons are of the same size as the corresponding exons of SLC4A1, and their overall genomic structures resemble each other (Medina et al. 1997). The SLC4A2 gene encodes a 1241 amino acid transmembrane protein, which differs mostly in its N-terminal region from that of SLC4A1 (Fig. 3).

The SLC4A3 gene was found in excitable tissues (Kopito et al. 1989; Su et al. 1994). The gene maps to 2q36, and encodes a 1232 amino acid transmembrane protein. SLC4A2 and A3 show stronger homology with each other than with SLC4A1. Furthermore, the N-terminus of SLC4A1 is ~300 amino acids shorter as compared to SLC4A2 and A3 (Fig. 3). Generally, the C-terminal membrane domains share the highest degree of amino acid identity (~67%) among the family members (Alper 1991).

The structure of the anion exchangers has been under extensive study for a long time. Most studies have focused on the SLC4A1 protein, which would serve as a prototype for the other members. Hydropathy analysis of the SLC4A1 amino acid sequence combined with experimental evidence derived from covalent labeling studies using impermeant reagents, proteolysis and antibody epitope mapping predicts topology models containing 12-14 membrane spans (Fig. 4) (Tanner 1993; Popov et al. 1997; Fujinaga et al. 1999; Popov et al. 1999).

At cellular level, the structure is still more complex, since SLC4A1 has been observed to form self-assembling oligomeric structures. The basic functional unit is homodimer (Wang et al. 1993). A three-dimensional map of the dimeric membrane domain has been determined using electron microscopy of two-dimensional crystals (Wang et al. 1994). The dimeric membrane domain shows a canyon-like structure, consisting of a basal domain and two large protrusions. However, structural details are limited to low-resolution (20Å), and further studies are required to understand their functional relevance.

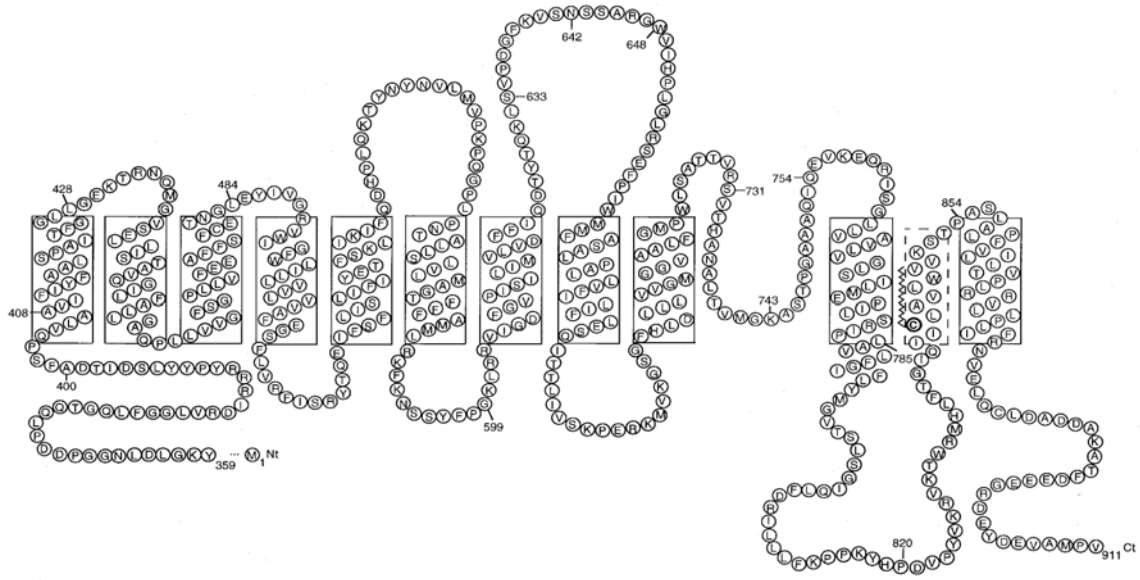

Figure 4. Predicted topology of the SLC4A1 protein (modified from Popov et al. 1998). The first 358 N-terminal amino acid residues have not been included in the figure.

2.1.2. Tissue distributions of SLC4A1-A3

Tissue distribution among the SLC4 family members varies considerably and reflects the tissue-specific functions of the proteins. Furthermore, the use of tissue-specific alternate promoters has been reported to all members (Linn et al. 1992; Sahr et al. 1994; Medina et al. 2000). Although, SLC4A1 is mainly expressed in red blood cells, it has been found also in many other tissues such brain, testis, lung, liver and gastrointestinal tract. An amino-terminal variant of SLC4A1 has been found in the basolateral membrane of type A intercalated cells of the renal cortical and medullary collecting ducts. This kidney-specific SLC4A1 lacks the first 65 residues of erythrocyte SLC4A1, which results from alternative promoter usage. Furthermore, a novel SLC4A1 isoform, nSLC4A1, has recently been cloned from rat heart, and proposed to be the predominant anion exchanger in cardiac ventricular myocytes (Alper et al. 1987; Brosius et al. 1989; Kudrycki and Schull 1989; Kudrycki et al. 1990; Kay et al. 1991; Puceat et al. 1995; Richards et al. 1999).

The SLC4A2 gene is transcribed in a wide variety of tissues and it has been present in almost all tissues examined so far. SLC4A2 is expressed in both epithelial and nonepithelial cells, and it is located mainly to the basolateral membrane of the cells. In human, the most abundant expression of SLC4A2 is seen in gastric parietal cells. Recently, the full-length isoform of SLC4A2 is localized to the Golgi, suggesting that it may play a role in the regulation of the anion exchange activity in intracellular membranes as well (Alper et al. 1988; Kudrycki et al. 1990; Lindsey et al. 1990; Alper 1994; Jons et al. 1994; Negrini et al. 1995; Wang et al. 1996; Holappa et al. 1999; Alferai et al.

2001; Holappa et al. 2001). Furthermore, five N-terminal SLC4A2 variants have been identified in mammals (Stuart-Tilley et al. 1998; Lecanda et al. 2000).

SLC4A3 is found at highest level in excitable tissues such as brain, heart and retina (Kopito et al. 1989; Linn et al. 1992; Kobayashi et al. 1994), but also in kidney, smooth muscle cells and throughout the gastrointestinal tract (Kudrycki et al. 1990; Brosius et al. 1997). Two different isoforms, bSLC4A3 and cA3, have been identified in heart, retina and smooth muscle cells (Linn et al. 1992; Kobayashi et al. 1994; Linn et al. 1995). cSLC4A3 contains 1034 amino acids, including the unique 73-amino-acid domain at the N-terminus (Yannoukakos et al. 1994). cSLC4A3 is most abundant variant expressed in heart, whereas in the retina bSLC4A3 and A3c are expressed at similar levels, but in different retinal cell layers. In addition, bSLC4A3 has been localized to both apical and basolateral membranes, whereas SLC4A3c localizes mainly to apical membranes (Alper et al. 1995).

2.1.3. Diverse biological functions of SLC4A1-A3

Besides regulating intracellular pH via the Cl^-/HCO_3^- exchange, the members of the SLC4 family exert various cellular functions including anchorage of the cytoskeleton to the membrane, regulation of metabolism, and generation of a senescence antigen. The first assignment of anion transport function for SLC4A1 was based on studies searching potent anion exchange inhibitors (Cabantchik 1990). Most importantly, SLC4A1 and carbonic anhydrase (CA) catalyze interconnected processes involved in blood gas exchange (Sly and Hu 1995). In the peripheral tissues, CO_2 diffuses into the erythrocytes and is hydrated by CA. The resulting bicarbonate is transported into the blood plasma by SLC4A1. In the lungs, the process is reversed: bicarbonate enters the red cell via SLC4A1 and is dehydrated by CA, and the resulting CO_2 diffuses out of the red cells. Each SLC4A1 protein has been proposed to transport ~100 000 pairs of monovalent anions per second, which is the largest ion-specific flux known for any single cell in the body (Weith et al. 1982).

Anion specificity of SLC4A1 includes also a wide range of oxyanions such as sulfate, selenate, selenite and phosphate (Galanter et al. 1993; Sekler et al. 1995; Dale et al. 1996). Transport activity can be inhibited by stilbene disulfonates, DIDS and SITS, which are known anion exchanger specific inhibitors (Salhany 1998). The transport function is related to the C-terminal membrane domain, which can be removed from the N-terminus without affecting the anion transport function (Grinstein et al. 1978). Interestingly, distinct functional features have been found in different

species: trout anion exchanger (tslc4a1), but not the mouse slc4a1, elicits, in addition to anion exchange activity, both anion conductance and transport of uncharged organic solutes such as taurine, urea and sorbitol (Fievet et al. 1998). Recently, the short acidic cytoplasmic domain in the ultimate C-terminus of SLC4A1 has been observed to bind to carbonic anhydrase II to promote bicarbonate transport across cell membranes (Vince and Reithmeier 2000).

In addition to ionic homeostasis, SLC4A1 provides the mechanical stability in erythrocytes, serving as a critical attachment site for different cytoskeletal components (Low 1986). A structural component of erythrocytes, spectrin, is able to bind to N-terminus of SLC4A1 via ankyrin and Band 4.1, making it essential for maintaining cell shape and mechanical strength (Bennett 1989; Davis and Bennett 1990). The highly acidic N-terminus of SLC4A1 participates also in glucose and oxygen metabolisms, since the N-terminus provides an association site for glycolytic enzymes, glyceraldehyde-3-phosphodehydrogenase (G3PDH), aldolase, phosphofructokinase as well as catalase, hemoglobin, hemichromes, band 4.1 and band 4.2 (Low 1986; Bennett 1989; Matayoshi and Jovin 1991). There is also evidence for glycophorin A (GPA) and SLC4A1 interaction during their biosynthesis and processing. In red blood cells lacking GPA, the average length of N-glycan chain attached to SLC4A1 is increased and the anion transport activity of SLC4A1 is decreased, suggesting that SLC4A1 is dependent on GPA for efficient translocation to the membrane and for final processing in order to adopt active conformation (Gahmberg et al. 1976; Groves and Tanner 1992). These results have been confirmed recently in SLC4A1 knockout mice, which indicate that GPA is not expressed in the red cell membranes in the absence of SLC4A1 (Hassoun et al. 1998). The kidney-specific isoform of SLC4A1 binds to the kanadaptin protein and has been suggested to regulate blood pH (Chen et al. 1998). The SLC4A1 protein has been suggested to act as a senescence antigen for aged and damaged red cells (Kannan et al. 1988)

Cl^-/HCO_3^- exchange mediated by SLC4A1 displays a broad pH versus activity profile, consistent with its role in facilitating the CO_2 metabolism. In contrast, SLC4A2 has been proposed to function as a highly pH-sensitive anion exchanger in different transport systems. It mediates Cl^- influx in *Xenopus* oocytes (Alper et al. 1989), and induces a DIDS-sensitive Cl^-/HCO_3^- exchange in various mammalian cell lines (Lindsey et al. 1990; Lee et al. 1991; Sekler et al. 1995). The activity of SLC4A2 can be enhanced at alkaline pH, consistently with the possible role of SLC4A2 in the control of intracellular pH (Lee et al. 1991; Humphreys et al. 1994; Humphreys et al. 1995; Humphreys et al. 1997). Furthermore, SLC4A2 exhibits several modes of regulation not displayed by SLC4A1: it can be inhibited by protons, activated by hypertonicity and ammonium ion, and

SLC4A2 can mediate regulatory volume increase in *Xenopus* oocytes, while SLC4A1 lacks these properties (Humphreys et al. 1994; Jiang et al. 1994; Humphreys et al. 1995; Humphreys et al. 1997).

Two different models have been proposed for the pH sensitive modulation of the SLC4A2 activity. In human embryonic kidney (HEK) 293 cell line, the possible "pH sensor" of SLC4A2 has been mapped to a cluster of four histidine residues located within the extreme N-terminus of SLC4A2 and A3, but absent from SLC4A1 (Sekler et al. 1996). In contrast, the *Xenopus* oocyte experiments suggested that the pH sensor of SLC4A2 is intrinsic to the membrane domain and is subject to modulation by a modifier site located within the N-terminal cytoplasmic domain (Zhang et al. 1996). These results were further defined within the NH_2-terminal domain, which demonstrated distinct structural requirements for SLC4A2 regulation by intracellular and extracellular protons (Stewart et al. 2001). In spite of the differences of the pH sensor site of SLC4A2, both models suggested that the pH-dependent modulation of the transport activity probably requires an interaction between the cytoplasmic N-terminus and the catalytically active C-terminal membrane domain (Sekler et al. 1996; Zhang et al. 1996).

The SLC4A3 protein functions also as a DIDS-sensitive, Na^+-independent anion exchanger, regulating both the intracellular pH and Cl^- gradients in excitable cells. Expression of SLC4A3 in COS cells demonstrated cytoplasmic acidification and led to chloride- and bicarbonate-dependent changes in intracellular pH (Kopito et al. 1989; Sterling and Casey 1999). The full-length SLC4A3, which is postulated to regulate the intracellular pH in glial cells, contains the pH sensor domain similar to SLC4A2 (Sekler et al. 1996). However, the SLC4A3c isoform that predominates in the heart lacks the N-terminal 270 amino acids that encompasses the pH sensor, consistently with its proposed role as a regulator of the Cl^- gradient rather than the pH_i in these cells (Kudrycki et al. 1990; Sekler et al. 1996).

2.1.4. Anion exchangers of the SLC4 family in pathophysiological states

The naturally occurring variants and mutations of SLC4A1 have been linked to different diseases such as Southeast Asian ovalocytosis, hereditary spherocytosis and distal renal tubular acidosis of both dominant and recessive forms (Bruce and Tanner 1999; Alper 2002). Furthermore, abnormal expression of SLC4A1 and A2 mRNAs has been observed in primary biliary cirrhosis (PBC), suggesting that they may have a role in cholestasis, secretory failure, and immunodysfunction of PBC (Prieto et al. 1993; Medina et al. 1997). An autosomal recessive neurological disease,

choreoachantocytosis, has been associated with the altered C-terminus of SLC4A1 (Kay et al. 1990; Kay et al. 1991). Abnormal SLC4A1 has been found also in brains from Alzheimer disease patients (Kay 1991). However, naturally occurring mutations for SLC4A2 and A3 have not been reported so far.

Expression of SLC4A1 in red cell membranes is important to the organization of the membrane layer, and the defective protein may affect the morphology and stability of the red cell membrane. Two different phenotypes have been linked to structural abnormalities including Southeast Asian ovalocytosis (SAO) and hereditary spherocytosis. SAO occurs mainly in Malesian populations, although it has also been found in some other countries (Bruce and Tanner 1999). The SAO phenotype is asymptomatic, but it has been associated with protection against cerebral malaria in endemic areas (Genton et al. 1995). SAO results from the deletion of amino acid 400-408 of SLC4A1 (Schofield et al. 1992), which alters the protein structure abolishing its transport function, binding to inhibitors and N-glycosylation (Sarabia et al. 1993; Chambers et al. 1999). SAO red cells have been shown to be more rigid than normal red cells, suggesting that the deletion abrogates the proper interaction of SLC4A1 with the cytoskeleton (Mohandas et al. 1992). The altered SLC4A1 in SAO forms heterodimers and affects the normal population of SLC4A1 in red cells (Jennings and Gosselink 1995), reducing the anion transport activity of red cells about 60% compared to that in normal cells (Schofield et al. 1992). All the known individuals with SAO have been heterozygotes for SLC4A1, suggesting that the homozygous condition is lethal (Liu et al. 1994).

Most of the SLC4A1 mutations have been linked to autosomal dominant hereditary spherocytosis (Hassoun and Palek 1996; Bruce and Tanner 1999). It is characterized by spherocytic red cells with increased osmotic and mechanical fragility and results in haemolytic anemia. The spherocytic shape of red cells results from the loss of surface membrane area relative to intracellular volume. HS missense mutations are distributed throughout the SLC4A1 cytoplasmic and transmembrane domains. Certain mutations associated with HS have been shown to affect the protein 4.2 and ankyrin binding sites on SLC4A1 disrupting the cytoskeleton (Rybicki et al. 1993; Inoue et al. 1998). However, most of the mutations reduce the amount of SLC4A1 in the red cell membrane due to an unstable mRNA or degradation and mistargeting of the protein. Subsequently, the decreased expression of SLC4A1 in red cells leads to a proportional reduction in protein 4.2 expression destabilizing the membrane lipid bilayer and resulting in HS (Lux and Palek 1996).

The majority of the SLC4A1 mutations reported to date cause only erythroid abnormalities without renal phenotype. However, some of the SLC4 mutations, which are restricted to transmembrane domain have been associated with both dominant and recessive distal renal tubular acidosis (dRTA) (Bruce et al. 1997; Jarolim et al. 1998; Karet et al. 1998; Tanphaichitr et al. 1998). Interestingly, HS- and dRTA-associated mutations segregate almost completely, and only in two cases have homozygous recessive SLC4A1 mutations associated with anemia and dRTA (Lima et al. 1997; Rysava et al. 1997; Ribeiro et al. 2000).

The spectrum of clinical severity in dRTA is wide and correlation of genotype and phenotype can vary within individual families (Weber et al. 2000). The main symptoms of dRTA patients are characterized by reduced ability to acidify urine leading to variable hypochloremic metabolic acidosis, which is usually accompanied by nephrocalcinosis, kidney stones and metabolic bone disease. However, alkali replacement can reverse most of the biochemical abnormalities. Some patients with dominant dRTA remain asymptomatic until adolescence or adulthood, whereas those with the recessive disease may be severely affected in infancy, with impaired growth and early nephrocalcinosis causing eventual renal insufficiency. Many patients with recessive dRTA have also sensorineural deafness (Battle et al. 2001).

Functional studies on the dominant dRTA-associated SLC4A1 mutations have exhibited only moderate or mild decrements in the protein function, and the abundance of SLC4A1 in red cell membranes has been normal (Jarolim et al. 1998). The first recessive dRTA mutation, G701D, produced a conditional trafficking phenotype. Its folding and trafficking was dependent on the presence of glycophorin A, and because intercalated cells in kidney lacks this protein, SLC4A1 G701D could not accumulate at the cell surface. Consistently, SLC4A1 content and function were normal in patient red cell, which express GPA (Alper 2002). In addition, some recessive and compound heterozygous mutations have been shown to exhibit reduced transport activity (Bruce et al. 2000). However, additional functional studies will be required to understand SLC4A1-associated dominant and recessive dRTA in the absence of erythroid disease.

The pathophysiology of the SLC4A1-related diseases has been assessed also in two mouse models by selectively inactivating the erythroid but not kidney SLC4A1 (Peters et al. 1996; Southgate et al. 1996). The SLC4A1 null mice showed retarded growth, spherocytic red blood cell morphology, and severe hemolytic anemia. The majority of homozygous mice died within 2 weeks after birth. The expression of ankyrin-1 was decreased ~50% in $SLC4A1^{-/-}$ membranes and SLC4A1-binding

proteins 4.2 and glycophorin A were missing (Hassoun et al. 1998). However, the $SLC4^{-/-}$ erythrocytes assembled a normal membrane skeleton, thereby challenging the previous hypothesis that the presence of SLC4A1 is essential for the stable biogenesis of membrane skeleton (Peters et al. 1996; Southgate et al. 1996).

In another study, the gene targeting produced a null mutation that disrupted both the red blood cell and kidney AE1 isoforms. This resulted in a hypercoagulable state due to disruption of the phospholipid asymmetry of the red blood cell membrane. Moreover, a widespread thrombosis in vivo was observed, with only 5-10% of mice surviving the neonatal period (Peters et al. 1996; Hassoun et al. 1998). Similar observations have also been found from cattle red blood cells with a naturally occurring homozygous nonsense mutation of the SLC4A1 gene (Inaba et al. 1996).

2.2. The SLC26 gene family defines a new group of anion exchangers

As the biochemical and biophysical studies on the red blood cell membrane paved the way for the exploration of the SLC4 gene family of anion exchangers, the identification of the second anion exchanger family was mainly based on the positional cloning of the genes for different genetic diseases. Strikingly, clinically distinct diseases, diastrophic dysplasia, congenital chloride diarrhoea and Pendred syndrome, bearing no obvious resemblance to one another turned out to be caused by defects in genes, which belong to the same gene family. The clearly distinguishable group of the previously so-called sulfate transporters was then recently redefined functionally as the second anion exchanger gene family, SLC26 (Kere et al. 1999).

2.2.1. Rat slc26a1 functions as a sulfate/anion exchanger in hepatocytes

The first member of the SLC26 family, named sulfate anion transporter-1, was isolated from a rat liver cDNA library by expression cloning, as a result from the search for novel mammalian sulfate transporter systems (Bissig et al. 1994). The rat slc26a1 cDNA of 3.7 kb encodes a protein of 703 amino acids. The protein sequence contains three putative N-glycosylation sites, several phosphorylation sites and 15 putative N-myristylation sites. Hydrophobicity analysis suggests 12 putative transmembrane segments with intracellular N- and C-termini. Northern analysis indicated that SLC26A1 is most abundant in the liver and kidney, and with weaker signals in the skeletal muscle and brain. More detailed analysis using immunohistochemistry localized slc26a1 to the basolateral membrane of the proximal tubules in kidney (Karniski et al. 1998). In rat brain, slc26a1 was located in hippocampus and cerebellum, suggesting that it may play a role in sulfate transport in neuronal or glial cells (Lee et al. 1999).

Functional expression of rat slc26a1 has established its function as a Na^+-independent sulfate transporter that is strongly inhibited by DIDS and oxalate, but not by succinate or cholate (Bissig et al. 1994). These properties correlate with the rat liver mRNA induced sulfate transport in oocytes, and the functional activities of the sulfate/bicarbonate exchanger in liver canalicular membrane vesicles (Meier et al. 1987; Palacin et al. 1990). Similar results were observed also with the rat kidney and brain injected mRNAs, confirming that slc26a1 transports sulfate, oxalate and bicarbonate (Markovich et al. 1994; Lee et al. 1999).

2.2.2. Defective sulfate transport of SLC26A2 causes skeletal dysplasias

The second member of the SLC26 family was found in a search for diastrophic dysplasia (DTD) gene. DTD (OMIM 222600) is an autosomal recessive chondrodysplasia characterized with short-limbed dwarfism, spinal deformation, and generalized dysplasia of joints (Walker et al. 1972). A linkage disequilibrium approach mapped the diseases to 5q31-q34, which included a candidate gene that was not expressed in fibroblasts derived from the DTD patients (Hästbacka et al. 1990). The novel clone shared a significant homology with the recently identified rat slc26a1, making it very interesting, because one of the histological abnormalities of DTD patients is a deficient staining of sulfated proteoglycans in cartilage (Scheck et al. 1978). Subsequently, several point mutations were found in the gene, confirming that it was responsible for DTD (Hästbacka et al. 1994). The gene became known as diastrophic dysplasia sulfate transporter, DTDST, which is currently designated as SLC26A2.

The human SLC26A2 is comprised of four exons and three introns spanning over 40 kb (Clines and Lowett 1996). The gene contains an open reading frame of 739 amino acids with a molecular weight of 82 kDa. The hydropathy analysis of the sequence predicts a membrane protein with 12 transmembrane segments with highly charged cytoplasmic domains at both N- and C-termini. Two potential N-glycosylation sites can be found from the extracellular loop. The protein sequence showed strong sequence similarity to rat slc26a1, suggesting a putative function for SLC26A2 (Hästbacka et al. 1994). The mouse slc26a2 gene (named st-ob for sulfate transporter in osteoblast) has been recently isolated from immature mouse fibroblastic cells (Kobayashi et al. 1998).

SLC26A2 is widely expressed, although cartilage is the only tissue affected by its defective function (Hästbacka et al. 1994; Satoh et al. 1998). By in situ hybridization and immunohistochemistry, SLC26A2 expression localized to the secretory structures of different cell

types such as fetal hyaline cartilage, adult bronchial cartilage, eccrine sweat gland, bronchial glands, placental villi, exocrine pancreas and colon (Haila et al. 2000; Haila et al. 2001). Furthermore, SLC26A2 has been suggested to function as a Cl^-/SO_4^{2-} exchanger, because it can induce a DIDS-sensitive Na^+- independent SO_4^{2-} transport that can be inhibited by chloride, thiosulfate and oxalate (Satoh et al. 1998). These results are also consistent with the defective sulfate uptake of DTD patient fibroblasts and chondrocytes (Hästbacka et al. 1994; Rossi et al. 1996).

Four recessively inherited chondrodysplasias has been associated with over 30 mutations found in the SLC26A2 gene: diastrophic dysplasia (DTD), atelosteogenesis type 2 (AO2), achondrogenesis type 1B (ACG-1B) and recessive multiple epipyseal dysplasia (rMED) (Rossi and Superti-Furga 2001). This skeletal dysplasia family ranges from a relatively benign condition to a severe derangement of skeletal development incompatible with life. The mutations likely reduce the rate of sulfate flux from the pericellular space into cytoplasm, and result in undersulfation of proteoglycans as the consequence of intracellular sulfate pool depletion, leading to different disease phenotypes. Genotype-phenotype correlations indicate that the amount of residual activity of SLC26A2 strictly modulates the phenotype from lethal ACG1B to nonlethal DTD. Homozygosity or compound heterozygosity for stop codons or transmembrane domain substitutions mostly result in ACG-1B, while other structural or regulatory mutations usually result in a less severe phenotype. Although the nature of the mutations at SLC26A2 appears to explain much of the phenotypic difference among the three diseases, other factors may also play a role (Hästbacka et al. 1994; Hästbacka et al. 1996; Superti-Furga et al. 1996; Superti-Furga et al. 1996; Karniski 2001).

DTD is a skeletal dysplasia associated with short stature, joint contractures, cleft palate, and characteristic clinical signs such as hitch-hiker thumb and cystic swelling of external ears (Horton et al. 1978; Horton 1996). Skeletal defects are clinically diagnosable early in fetal development (Kaitila et al. 1983). Patients are severely handicapped and need repeated corrective surgery, but their intelligence and sexual development are within the normal range. In infancy, respiratory and spinal abnormalities lead to increased mortality, but thereafter life span is not remarkably decreased (Walker et al. 1972).

AO2 (OMIM 256050) is a more severe, neonatally lethal chondrodysplasia whose clinical and histological characteristics resemble those of the much less severe diastrophic dysplasia. Patients die of respiratory insufficiency shortly after birth because of the collapse of the airways and

pulmonary hypoplasia due to the small rib cage (Sillence et al. 1987). ACG-1B is lethal and characterized by extremely short limbs and characteristic radiographic appearance (Borochowitz et al. 1988). Affected individuals are usually born before term as stillborns or die immediately after birth. The latest addition to the SLC26A2 skeltal dysplasia family is a condition with clubfoot and mild finger deformity but absence of palatal clefting, ear swelling, or thumb deviation, and mildly shortened or normal stature, which has been classified as recessively inherited multiple epiphyseal dysplasia, rMED (OMIM 226900) (Superti-Furga et al. 1999; Superti-Furga et al. 2000).

2.2.3. SLC26A3 is an intestinal anion exchanger defective in chloride diarrhoea

The second member of the human SLC26 family was found by positional cloning of the congenital chloride diarrhoea (CLD) gene (Kere et al. 1993; Höglund et al. 1996). Studies with Finnish CLD families mapped the disease to 7q31-q32 and identified a disease gene, DRA, mutated in CLD (Kere et al. 1993; Höglund et al. 1996). The CLD gene has been recently symbolized as SLC26A3.

The SLC26A3 gene was originally isolated from a normal colon tissue cDNA library as a tumor suppressor candidate gene down-regulated early in tumorigenesis (Schweinfest et al. 1993). It comprises 39 kb of DNA and contains 21 exons (Haila et al. 1998). Its mRNA includes an open reading frame of 764 amino acids, encoding a 10-, 12- or 14-transmembrane glycoprotein (Fig. 5). The overall amino acid sequence homology is ~50% compared to SLC26A2. The deduced protein structure resembles a typical ion transporter molecule, being composed of multiple hydrophobic transmembrane domains with cytoplasmic N- and C-termini. The SLC26A3 protein may be variably N-glycosylated and subject to posttranslational cleavage near the amino-terminal end of the protein (Byeon et al. 1996; Byeon et al. 1998).

SLC26A3 is expressed mainly in the apical surface of enterocytes in duodenum, ileum and colon (Byeon et al. 1996; Höglund et al. 1996; Haila et al. 2000; Jacob et al. 2002), but also in other extraintestinal tissues including eccrine sweat glands, intraprostatic seminal vesicles and trachea (Haila et al. 2000; Wheat et al. 2000). In addition, SLC26A3 expression pattern is suggested to be dependent on the differentiation status of the tissue (Antalis et al. 1998; Haila et al. 2000). In inflammatory bowel disease and ischemic colitis, expression of SLC26A3 mRNA remains similar to histologically normal colon epithelium, but the protein localizes deeper in crypts (Antalis et al. 1998; Haila et al. 2000).

Heterologous expression systems have established SLC26A3 as an anion exchanger that transports SO_4^{2-}, Cl^- and oxalate (Silberg et al. 1995; Byeon et al. 1998; Melvin et al. 1999; Moseley et al. 1999). The SLC26A3 cRNA injected *Xenopus* oocytes demonstrated Na^+-independent DIDS-sensitive sulfate and chloride uptake. Similarily, Sf9 insect cells infected with the SLC26A3 recombinant baculovirus induced increase in sulfate uptake. The mouse slc26a3 functions as a Cl^-/HCO_3^{2-} exchanger with features compatible also with a Cl^-/OH^- exchanger (Melvin et al. 1999). Furthermore, the apical anion exchanger in rabbit and rat duodenum has recently been shown to mediate DIDS-sensitive Cl^-/Cl^-, HCO_3^{2-}/Cl^-, $SO_4^{2-}{}_i/Cl^-_o$ and $Cl^-_i/SO_4^{2-}{}_o$ exchange (Jacob et al. 2002), corresponding well with the transport characteristic of SLC26A3. Altogether, these findings demonstrate that SLC26A3 mediates an apical electroneutral Cl^-/HCO_3^{2-} exchange in different tissues.

In CLD, the Cl^-/HCO_3^- exchange is absent or defective, causing a severe intestinal Cl^- absorption defect (Holmberg et al. 1975). Large amounts of Cl^- are lost in the stools, and the patients develop hypochloremia. The respective defect in HCO_3^- secretion leads to metabolic alkalosis and the acidification of intestinal content, which further inhibit the absorption of Na^+ through Na^+/H^+ exchanger. In the intestine, the high luminal electrolyte contents leads to diarrhoea by osmotic mechanisms. Na^+ and water losses cause secondary hyperaldosteronism and K^+ wastage, leading to both hyponatremia and hypokalemia (Holmberg 1986).

Congenital chloride diarrhoea is a recessively inherited disorder of intestinal electrolyte absorption involving Cl^-/HCO_3^- exchange (Darrow 1945; Gamble et al. 1945). The main clinical feature of CLD is prenatal onset of watery diarrhoea, leading to polyhydramnios prematurity (Holmberg et al. 1975). Newborn babies have distended abdomen, absence of meconium, chronic diarrhoea and they fail to thrive. They develop hyperbilirubinemia, dehydration and metabolic alkalosis, and severe electrolyte disturbances with hyponatremia, hypokalemia and hypochloremia. The disease is often fatal if untreated by oral compensation of fluid and electrolyte losses (Holmberg 1986).

Since the identification of the SLC26A3 gene, 27 different mutations have been found in different populations and sporadic cases. The spectrum of the mutations includes transversions, transitions, insertions, deletions and two genomic rearrangements (Höglund et al. 1996; Etani et al. 1998; Höglund et al. 1998; Höglund et al. 1998; Höglund et al. 2001). In Finland and Saudi-Arabia, a single major mutation in each country (V317del and G187, respectively) has been shown to account for over 90% of the CLD-associated chromosomes (Höglund et al. 1998). However, the functional

characterization of the mutants remains still to be determined. Only the Finnish mutation, V317del, has been confirmed by a functional analysis in *Xenopus* oocytes, demonstrating a defective chloride and sulfate transport (Moseley et al. 1999).

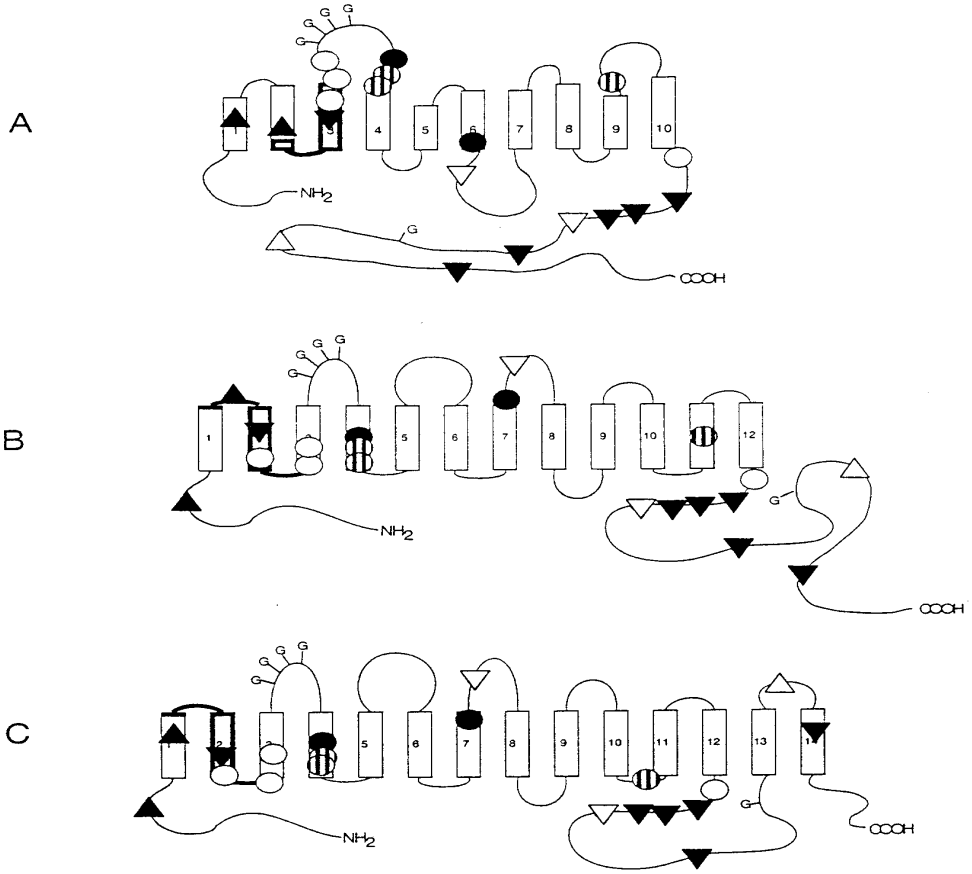

Figure 5. Predicted domain structures of the SLC26A3 protein and distibution of mutations. Three alternative models with 10 (A), 12 (B) or 14 (C) transmembrane segments have been presented. Location of some of the known mutations have been indicated with symbols as follows: point mutations are circles, insertions are triangles pointing upward and deletions are triangles pointing down, missense mutations are indicated with black symbols and splice site mutations with striped symbols. Potential N-linked glycosylation sites are indicated by G (Kere et al. 1999).

2.2.4. Defects in SLC26A4 chloride/iodide exchanger cause deafness with goitre

Pendred syndrome is an autosomal recessive disorder of congenital sensorineural hearing loss often with goitre (Pendred 1896). The deafness is present typically at birth, but can be variable in its expression, sometimes appearing during childhood. The hearing loss is generally profound and Pendred syndrome patients have structural malformations of the inner ear, the most common feature

of which is an enlarged endolymphatic duct (Cremers et al. 1998; Usami et al. 1999; Reardon et al. 2000). Even more variable in its presentation is the thyroid disease, goitre. It can develop at any age, but may be totally absent in some affected individuals (Johnsen et al. 1986). The goitre is often associated with an abnormal release of iodide from the thyroid, which can be analysed by a perchlorate discharge test (Sheffield et al. 1996).

Since the syndrome has been suggested to account for 10% of hereditary hearing loss, the identification of the Pendred syndrome gene has been of special interest (Fraser 1965). The identification of the gene was succeeded by positional cloning efforts (Coyle et al. 1996; Sheffield et al. 1996; Everett et al. 1997). Finally, the identification of independent mutations confirmed that Pendred syndrome is caused by mutations in a single gene, named PDS (Everett et al. 1997). The PDS gene consists of 21 exons, encoding a 780-amino acid transmembrane protein, pendrin. The PDS gene has been positioned tail-to-tail to the SLC26A3 gene on 7q22-q3.11. Its sequence is well conserved with the SLC26A2 and A3 sequences suggesting both evolutionary and functional relationships. Currently, the PDS gene has been designated as SLC26A4.

Expression of SLC26A4 is thought to be responsible for the iodide transport in the thyroid as well as the formation and function of the inner ear. In human, SLC26A4 is expressed at highest level in thyroid tissue, but it has been detected also in kidney, brain, placenta, cochlea, endometrium, lung, breast, prostate and testis (Everett et al. 1997; Everett et al. 1999; Royaux et al. 2000; Lacroix et al. 2001; Royaux et al. 2001; Suzuki et al. 2002). In thyroid gland, SLC6A4 is present exclusively in the apical membrane of the follicular epithelium in is regulated by thyroglobulin (Royaux et al. 2000). In the mouse inner ear, SLC26A4 has been localized to discrete areas, including regions thought to play a key role in fluid transport (Everett et al. 1999). Thus, the expression pattern of SLC26A4 points to a likely role for SLC26A4 in the thyroid and inner ear physiology defective in Pendred syndrome.

Heterologous expression systems have revealed that SLC26A4 can transport several different anions such as chloride, iodide, formate and nitrate, but not sulfate (Kraiem et al. 1999; Scott et al. 1999; Bogazzi et al. 2000; Scott and Karniski 2000). Specifically, iodide is taken up by thyroid via the sodium/iodide symporter (NIS) in the basolateral membrane of follicular thyrocyte (Fig. 6). Subsequently, it has been postulated that iodide is transported across thyrocyte´s apical membrane by SLC26A4 into the colloid and is then incorporated into thyroglobulin. Therefore, the functional activities of SLC26A4 correlate closely with previous findings showing that iodine organification

has been altered in thyroid cells obtained from a Pendred syndrome patient. Defective iodine organification results in goitre, with various degrees of hypothyroidism (Scott et al. 1999). However, recent expression and mutational studies point toward some redundancy in the role of SLC26A4 in the thyroid. It has been shown that SLC26A4 content of thyroid tissue does not vary with the functional activity of the gland in terms of iodine accumulation and utilization (Porra et al. 2002). Furthermore, some mislocalizing SLC26A4 mutants lacking the iodide transport function, do not associate with the thyroid pathology (Taylor et al. 2002), suggesting that there may be an alternative iodide transporter to facilitate efflux across the apical membrane of the thyroid.

Recently, SLC26A4 has also been proposed to function as a Cl^-/OH^- and Cl^-/HCO_3^- exchanger in cortical part of the kidney. However, PS patients have not reported to develop overt acid-base disturbances or other renal manifestations, suggesting compensatory activities of other transporters in kidney (Royaux et al. 2001; Soleimani et al. 2001).

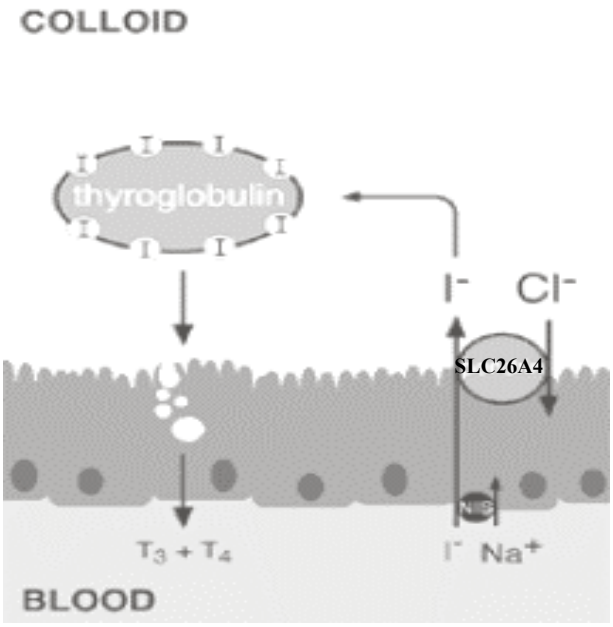

Figure 6. SLC26A4 functions as a Cl^-/I^- exchanger in thyroid tissue (modified from Everett and Green 1999).

More than 50 independent SLC26A4 mutations, distributed throughout the coding sequence, have been characterized as causing Pendred syndrome and nonsyndromic deafness (Everett et al. 1997; Coyle et al. 1998; Li et al. 1998; Coucke et al. 1999; Usami et al. 1999; Reardon et al. 2000; Campbell et al. 2001; Gonzalez-Trevino et al. 2001; Lopez-Bigas et al. 2001). Most mutations are missense, with a smaller number of insertions, deletions, frameshift, and splice site mutations. Although most mutations are related to Pendred syndrome (Coyle et al. 1998; van Hauwe et al. 1998), some of mutations associate with deafness without goitre, suggesting that the gene can

account for a broader spectrum of deafness than just Pendred syndrome (Usami et al. 1999). This notion is further supported by the presence of residual SLC26A4 function associated with some of mutations in deaf patients without goitre (Scott and Karniski 2000).

Targeted disruption of mouse slc26a4, which removed exon 8 of the gene, has also provided important clues about the etiology of deafness in Pendred syndrome (Everett et al. 2001). The possible role of SLC26A4 in the inner ear has been unclear, and it has been hypothesized that the chloride transport function of SLC26A4 is important in the homeostasis of the endolymph with ionic imbalance potentially responsible for auditory developmental malformation. The $slc26a4^{-/-}$ mice develop early-onset, profound deafness and display structural malformations in the inner ear, similar seen in Pendred syndrome patients. The inner ears of these knockout mice manifest severe endolymphatic dilatation, degeneration of sensory hair cells as well as other malformations. However, the $slc26a4^{-/-}$ mice showed a complete absence of any thyroid pathology, resembling patients with non-syndromic deafness associated with PDS-mutations (Everett et al. 2001).

2.2.5. The SLC26 family is structurally different from the SLC4 family

The SLC26 family is structurally different from the SLC4 family. Although the genomic structures with about 20 exons resemble each other, the proteins differ completely at amino acid level: any apparent homologous regions cannot be found. In addition, the amino acid sequences of the SLC4 members are nearly two times longer than those of the SLC26 proteins.

The overall secondary structure of the proteins resembles more each other, since both families encode typical ion transporters with a hydrophopic membrane domain of 10-14 transmembrane segments associated with the hydrophilic N- and C-terminal tails. However, SLC4 members have a large interactive cytoplasmic N-terminus and only a short cytoplasmic C-terminus, while the situation is conversed in the SLC26 family. They contain usually a short N-terminus and a large hydrophilic C-terminus, which provides interaction sites for cytoplasmic proteins. Furthermore, the SLC4 proteins have been observed to function in oligomeric forms and heterozygous mutations have been associated with diseases. In contrast, the members of SLC26 family are all related to recessive diseases and the heterozygotes carriers have been asymptomatic, suggesting that the basic functional unit is monomeric.

Despite these many structural differences, both families share a significant functional identity. The proteins are able to induce a Na^+-independent anion exchange activity, and their anion specificity

and sensitivity for inhibitors resemble also each other. These findings raise an intriguing question about the fundamental structural features required for anion transport beyond these two structurally different families. Comparison of the structure-function relationships as well as the regulation of the proteins utilizing naturally occurring and created mutants will help to answer the question. Moreover, identification of novel structural homologs with new properties or diseases associations might also help to understand the anion transport physiology.

In a search for the new SLC26 members, we utilized the rapidly accumulating genomic sequences in databases (Kere et al. 1999). The sequences of the first eukaryotic species, *Saccharomyces cervisiae* and *Chaenorhabditis elegans* had recently been annotated in public databases. A homology comparison with known family members revealed that the SLC26 family forms a large group of multispecific carriers present among bacteria, yeast, fungi, plants and animals (Kere et al. 1999). Of interest, seven different genes were identified in plants such as *Arabidopsis thaliana* and animals such as *C. elegans*. Thus the human SLC26 gene family with three known members was likely to expand, and the still unknown genes might relate to new physiological processes or diseases.

3. AIMS OF THE CURRENT STUDY

A growing interest in the SLC26 family was stimulated by the involvement of the first three members, SLC26A2-A4, in phenotypically different recessive diseases. Futhermore, a homology search revealed that SLC26 family is well conserved across taxonomic span, including seven putative members in the first fully sequenced animal, *C. elegans*. This prompted us to hypothesize that more than three genes with important physiological functions or disease associations might exist also in human. The principal goal of this study was thus to expand the SLC26 family and to characterize its novel members.

In addition, as an example of the physiological role of ion transporters in pathophysiological states, we analysed how intestinal inflammation affects the expression of three major transporters of intestinal salt transport, SLC26A3 (Cl^-/HCO_3^--exchanger), SLC9A3 (Na^+/H^+-exchanger) and CFTR (Cl^--channel), to understand the pathophysiology of diarrhoea in ulcerative colitis. In inflamed colonic mucosa, the equilibrium between absorptive and secretory functions for electrolyte and water transport is disturbed. All three transporters do likely play in concert with each other, and their functional defects have been associated with diarrhoeal diseases. Therefore, putative changes in the expression or function of the transporters might contribute to diarrhoea, which is a prominent symptom of ulcerative colitis.

These starting points of the study led to the following aims:

- to identify and map new candidates to the SLC26 family
- to clone and characterize the new genes
- to establish the expression pattern and the preliminary function of the new genes
- to analyse the effects of intestinal inflammation on expression of SLC26A3, SLC9A3 and CFTR in ulcerative colitis

4. MATERIALS AND METHODS

4.1. Computational sequence analysis (I-III)

New homologs were searched by the human SLC26A2, SLC26A3, SLC26A4 nucleotide and protein sequences against different databases with BLASTP, BLASTX, TBLASTN, TBLASTX and FastA algorithms (Pearson 1994; Altschul et al. 1997; Zhang et al. 1997). GENSCAN, FGENES, and GRAIL2 were used to predict the coding exons from genomic clones (Xu et al. 1994; Solovyev et al. 1995; Burge and Karlin 1997). Multiple sequence alignment was done by ClustalW (Thompson et al. 1994) and GENEDOC programs (http://www.cris.com/ ~Ketchup /genedoc.shtml) or by ClustalX and BoxShade Server 3.21 (http://www.ch.embnet.org/ software/ BOX_form.html). Transmembrane topologies of the SLC26A6-A9 proteins were predicted by the TMHMM (Krogh et al. 2001) and PSIpred programs (Jones et al. 1994). Putative N-glycosylation sites were analysed by the PROSITE program (Bairoch et al. 1997).

4.2. Chromosomal mapping by radiation hybrids (I)

Chromosomal locations were assigned by PCR with gene specific primers using the GeneBridge 4 Radiation Hybrid and Stanford G3 panels (Research Genetics, Inc, Huntsville, Alabama). PCR products were run on 2% agarose/EtBr gel and results were analysed according to the publicly available RH-mapper servers at http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl and http://www-shgc.stanford.edu/RH/rhserver formnew.html.

4.3. Rapid amplification of cDNA ends, PCR assays and sequencing (I-III)

The coding regions of each gene were assembled from several overlapping PCR fragments, which were amplified by gene specific primers designed to GENSCAN-predicted exons (for details see Table 5 below). The overlapping fragments were amplified by PCR from the first strand cDNAs of human kidney, testis and lung. The first strand cDNAs were synthesized from 1 μg poly(A)$^+$ RNA (Clontech, Palo Alto, USA) by RT-PCR using the SMART RACE cDNA Amplification Kit (Clontech). The SMART RACE method was used for amplification of the 5´ and 3´ ends of the cDNAs. Furthermore, the cloning of the 3´ends of the genes was also accomplished by PCR with primers designed to the regions, which matched the expressed sequence tags (EST) annotated in GenBank. PCR products were run on agarose gels, excised and purified for sequencing or subcloned by TOPO-TA system (Invitrogen) before sequencing. The PCR products were sequenced using dye-terminator chemistry (Sanger et al. 1977) and an automated sequencer (ABI 373A, Applied Biosystems).

4.4. Polyacrylamide gel electrophoresis analysis of single residue polymorphism (I)

During the cloning of the SLC26A6 gene several alternative sequences were found, suggesting alternative splicing events. First strand cDNA was synthesized from 1 μg of human kidney total polyA RNA (Clontech) by the SMART RACE cDNA Amplification Kit (Clontech) according to manufacturer´s instructions. To separate a putative splicoform of SLC26A6, missing three base pairs in the beginning of exon 17, the region was amplified by PCR with exon 16 and 17 specific primers. The PCR products were separated on a 6% polyacrylamide gel and visualized by silver staining. The polymorphism was also verified by cloning and sequencing the PCR fragments. One hundred genomic control DNA samples were analysed in the same way to exclude possible genetic polymorphism as a reason for variation.

4.5. Expression analysis from cell lines (I-III)

The expression of SLC26A6 and SLC26A9 genes was analysed from different cell lines by RT-PCR. Expression of SLC26A6 was analysed from human embryonal kidney (HEK) 293 and two human pancreatic duct cell lines, Capan-1 and Capan-2 (ATCC, Manassas, USA) and the expression of SLC26A9 from two human lung-specific epithelial cell lines, NCI-H358 and A549, from bronchoalveolus and alveolus, respectively. The HEK 293, NCI-H358 and A549 cells were grown in Dulbecco´s Eagle Medium (Gibco BRL, Gaithersburg, MD) with 50 U/ml penicillin, 2 mM sodium pyruvate, 2 mM glutamine and 5% fetal bovine serum. Capan-1 cells were grown in Iscove´s modified Dulbecco´s medium with 4 mM L-glutamine, 50 μg/ml penicillin and 20% fetal bovine serum and Capan-2 cells in McCoy´s 5a medium with 1,5 mM L-glutamine, 5050 μg/ml penicillin and 10% fetal bovine serum. The cells were grown at 37°C in 5% CO2 atmosphere to achieve full confluence. Total RNAs were isolated according to QIAGEN RNA Isolation Kit and the first strand cDNAs were synthesized from 1-2 μg of total RNA by the SMARTRACE cDNA Amplification Kit (Clontech). The SLC26A6 and A9 genes were amplified by PCR with gene specific primers and analysed by running on an agarose gel. The identity of PCR products was verified by sequencing.

4.6. Expression analyses by Northern blots and PCR panels (I-III)

Northern analysis was done using the Clontech (Palo Alto, USA) MTN Northern blots (MTN 7780-1 and MTN 7760-1). A1984-bp PCR amplified probe corresponding to nt 198 to 2181 of SLC26A7, a 551-bp probe corresponding to the 5´sequence from nt 156 to 707 of SLC26A8 and a 2362-bp

probe corresponding to the open reading frame sequence from nt 115 to 2476 of SLC26A9 were radiolabelled with ^{32}PdCTP with Rediprime Kit (Amersham) according to manufacturer´s instructions. The specific probes were hybridized to filters for different periods, and after washes the results were visualised by autoradiography performed on X-ray films. PCR analyses were done using Clontech´s multiple tissue cDNA panels I and II (K1420-1 and K1421-1) with the gene specific primers. The PCR results were analysed on agarose gel.

4.7. In situ hybridization (I-II)

Formalin fixed, paraffin-embedded specimens of normal adult human kidney and testis were obtained from the Department of Pathology, Haartman Institute, University of Helsinki. A 471-bp fragment corresponding to nucleotides 621-1091 of SLC26A6 cDNA and a 538 bp fragment corresponding to positions 1629-2166 of the SCL26A8 cDNA were amplified by PCR and used to transcribe sense and anti-sense RNA probes (Saarialho-Kere et al. 1994). All sections were pretreated with 1 μg/ml of proteinase K and washed in 0,1 M triethanolamine containing 0,25 % acetic acid. Sections were hybridized with ^{35}S-labeled probes (4 x 10^4 cpm/ml of hybridization buffer) at 52-55°C in a humidified chamber for at least 18 hours. Slides were then washed under stringent conditions, including treatment with RNAse A to remove unhybridized probe (Prosser et al. 1989) before dipping to LM-1 emulsion (Amersham, Buckinghamshire, England). After 10-54 days of autoradiography, the photographic emulsion was developed, and the slides were counterstained with hematoxylin and eosin. The sense RNA probe was used as a negative control.

4.8. Antibodies (I-IV)

Specific antibodies were raised in rabbits against the amino-terminal, MDLRRRDYHMERPLLN QEHL, and carboxy-terminal amino acids, TFALQHPRPVPDSPVSVTRL, corresponding to the nucleotides 252-312 and 2405-2465 of the SLC26A6 cDNA sequence (AF279265), respectively. Similarily, antisera were raised also against the synthetic peptides SHIHSNKNLSKLSDHSEV for SLC26A7, VEEVWLPNNSSRNSSPGLPD for SLC26A8 and ELSLYDSEEDIRSYWDLEQE for SLC26A9, corresponding to amino acids 639-656, 680-699 and 758-777, respectively. Two rabbits were immunized per each peptide and peptide synthesis and antibody production were purchased from Sigma-Genosys Ltd (London Road, Pampisford, Cambridge). SLC26A6 antibodies were purified from whole serum by affinity chromatography with the peptide coupled to *N*-hydroxysuccinimide-Sepharose 4B according to the manufacturer's instructions (Amersham Pharmacia Biotech). The C-terminal antiserum was raised in rabbits against the synthetic peptide corresponding to nucleotides 2375-2416 of the published SLC26A3 sequence (GenBank

L02785)(Research Genetics, Huntsville, Ala., USA). The cloned region was produced and purified as GST (glutathione-S-transferase) fusion protein according to the manufacturer's instructions (Amersham Pharmacia Biotech). The purified fusion protein was used then as antigen in the immunization of two rabbits (Animal Core Facility, Viikki Biocenter, University of Helsinki). Preimmune sera were collected before the first immunizations. A total of five immunizations were performed at three weeks´ intervals using ~400 µg of the GST fusion protein mixed 1:1 with Freund´s adjuvant. After the immunization, rabbits were bled and the sera were divided to small aliquots for preservation at -20°C. The specificity of the anti-SLC26A6 antibodies was analyzed by western blotting with SLC26A6-transfected COS-1 cells, and by in vitro translation of the protein according to the manufacurer´s instructions (TNT T7 Quick Coupled Transcription/Translation System, Promega). COS-1 cells grown on 6 cm plates were transiently transfected using Fugene6 liposomes (Roche Molecular Biochemicals) with either the SLC26A6a clone (5 µg) or water as a control, following manufacturer´s instructions. Three days after the transfections, the cells were lysed in 500 µl of boiling Laemmli sample buffer (Pharmacia) with 5% β-mercaptoethanol and separated by running on a 9% polyacrylamide gel before transferred electroforetically to a Hybond C-extra membrane (Amersham). The non-specific protein binding sites were saturated with 5% milk in PBS, 0.2% Tween-20 before the proteins were detected by the antibodies. Appropriate dilutions of the primary antibodies were determined by immunoblotting. Horseradish peroxidase conjugated goat anti-rabbit IgG (Sigma) was used as a secondary antibody. Preimmune sera in the same concentrations were used as controls. The protein bands were visualized by chemiluminescent reaction and the signals were recorded by exposure to an X-ray film (Fuji).

4.9. Immunohistochemistry (I,II,IV)

Formalin fixed, paraffin-embedded specimens of normal adult human kidney and pancreas were used for immunohistochemistry. For pre-treatment, deparaffinized slides were treated at 100°C in microwave oven in 10 mM citrate buffer (pH 6.0) for 8 min and endogenic peroxidase activity was inactivated by 0.3% H_2O_2 /metanol at 25°C for 30 min. Appropriate dilutions of the anti-SLC26A6-A9 or CFTR sera were used (diluted 1:50-1:2000), and immunostaining was done according to the VECTASTAIN Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA). Diaminobenzidine (DAB) was used as the chromogenic substrate and slides were counterstained by Mayer´s hematoxylin. Preimmune serum was used as negative control for parallel sections.

4.10. Functional transport measurements in *Xenopus laevis* oocytes (II-III)

The animal experiments were performed by the author in the laboratory of Dr. Daniel Markovich, University of Queensland, Brisbane, Australia. Mature *Xenopus laevis* females were purchased from the African *Xenopus* Facility C.C., Noordhoek, South Africa. Stage V and VI oocytes from *X. laevis* were maintained at 17°C in modified Barth's solution (88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, 10 mM HEPES/Tris, pH 7.4, gentamycin sulfate 20 mg/l). Oocytes were injected with either 50-100 nl of water (control) or 7-12 ng of SLC26A7-A9 cRNA using a Nanojet automatic injector (Drummond Scientific Co., Broomall, PA, USA). The cDNA sequences of the SLC26A6a, SLC26A6c, SLC26d, SLC26A6adel and SLC26A6cdel were amplified by PCR and subcloned to pCRII plasmids (Invitrogen).

A specific antisense primer (5'-CTA GAC CGA AAC AGG GCT GTC GGG GAC-3') missing the last nine bases but including a native stop codon was generated to delete the PDZ interaction motif (TRL) from SLC26A6adel and SLC26A6cdel contructs. The clones were verified by sequencing. For cRNA synthesis, plasmids were linearized by NotI digestion and transcribed in vitro using T7 RNA polymerase (Promega) and the resulting capped cRNAs were resuspended in water before use. Similarly, for cRNA synthesis, pcDNA3.1:SLC26A7 or pcRII:SLC26A7, pcDNA3.1:A8 or pNKS2:SLC26A8 and pcRII:SLC26A9 plasmids were linearized by EcoRV, NotI or XhoI digestions; the cDNAs were *in vitro* transcribed using T7 or SP6 RNA polymerases (Promega) and the resulting capped cRNA was dissolved in MilliQ water before use. Transport of ^{35}S-sulfate, $^{36}Cl^-$-chloride and ^{14}C-oxalate uptake was performed three days after injection. Briefly, 10 oocytes (per data point) were washed at room temperature for 1-2 min in solution A (115 mM sodium gluconate, 2.5 mM potassium gluconate, 4 mM calcium gluconate, 10 mM HEPES/Tris pH 7.4) or solution B (100 mM choline chloride, 4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 20 mM Hepes/Tris, pH 7.5), then placed into 100µl of solution A containing 2.5 mM NaCl with $^{36}Cl^-$ or 0.1 mM oxalate with ^{14}C-oxalate 2-5 µCi/ml, or into 100 µl solution B containing 0.1 mM K_2SO_4 with $^{35}SO_4^{2-}$ 10 µCi/ml or (New England Nuclear Radiochemicals) for 30-60 min at room temperature. The oocytes were washed 4 times with ice-cold solution B, lysed with 4% SDS, dissolved in scintillant (BCS, Amersham) and counted by liquid scintillation spectrometry. Inhibition of the sulfate uptake of SLC26A6 isoforms was performed by adding 1 mM DIDS to the uptake solution B. For the bicarbonate experiment, the standard 100mM NaCl uptake solution was replaced with 100 mM Na bicarbonate. Inhibition of the sulfate uptake of SLC26A9 was performed by adding either 1 mM DIDS, 5 mM thiosulfate, 5 mM oxalate or 5 mM glucose to the uptake solution B. All isotopes

were purchased from New England Nuclear Radiochemicals. Statistical analyses of the transport results were performed using the prism statistic package, version 3.0 (Graphpad software Inc. San Diego, California, USA). The degree of statistical significance between two groups was calculated using the unpaired t-test, with $p<0.05$ considered significant.

4.11. RNase protection analysis (III)

The presence of the SLC26A6 isoforms was analyzed by the ribonuclease protection technique according to the Direct Protect kit (Ambion Inc., Austin, TX) with two different RNA probes. A PCR fragment of 300 bp (bases 774-1075 in SLC26A6 cDNA, GenBank AF297659) to detect the SLC26A6a and A6c, and another fragment of 363 bp (bases 2085-2316 +131 bp from the end of intron 16 to detect SLC26A6a and A6d were amplified from human kidney cDNA (Clontech), purified and subcloned to pCRII-TOPO (Invitrogen) to utilize the T7 and SP6 priming sites in the generation of the transcription templates. The orientation of the fragments was verified by sequencing. The transcription templates were amplified by PCR using the same forward primers as used in the previous PCR with T7 or SP6 oligo as an antisense primer. Antisense RNAs of 396 nt (probe 1) and 446 nt (probe 2) were transcribed with T7 or SP6 RNA polymerase by the Maxiscript SP6/T7 Kit (Ambion) in the presence of [α-^{32}P]UTP, and hybridized with 10 µg of total, 1 µg polyA human kidney RNA, or 1 µg polyA human lung RNA. After an overnight hybridization, unpaired RNA was degraded by treatment with RNase Cocktail at 37°C for 30 min followed by isopropanol precipitation. Protected RNA fragments were fractionated by 5% SDS-PAGE containing 6 M urea and visualized by autoradiography.

4.12. Cell transfection and immunofluorescence (III)

Topologies of the SLC26A6 isoforms were analyzed in transfected permeabilized and non-permeabilized COS-1 cells by immunofluorescence microscopy. The cDNAs of the SLC26A6a, SLC26A6c and SLC26d were amplified by PCR and subcloned to eukaryotic pcDNA3.1/V5/His-TOPO plasmid (Invitrogen). The same primers were used for template production as in the splicing analysis. For immunofluorescence staining, COS-1 cells plated on glass coverslips were transiently transfected using Fugene6 (Roche Molecular Biochemicals) with either the clones or water as a control, following manufacturer´s intructions. The cells were grown as described above. After 48 h, the cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS, 0.14 M NaCl in 10 mM sodium phosphate buffer, pH 7.4). After fixing, the coverslips were washed two times with PBS and permeabilized with 0.1% Triton in PBS 30 min continuing then with blocking. If not permeabilized, the cells were washed after fixation and then blocked with 3% goat serum in PBS

for 1 h. Affinity-purified antibodies were then added (1-5 μg/ml) in 1% goat serum in PBS and incubated at room temperature for 1 h. After three washes with 3% goat serum in PBS, fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgGs (Sigma) were added and incubated for 1 h. Coverslips were then washed 5 times with PBS and mounted on glass slides using Immu-mount medium (Shandon, Pittsburgh, PA). Sections were examined and images were acquired on a Leica immunofluorescence microscopy.

4.13. In vitro binding assays (III)

The protein binding assays were performed in the laboratory of Dr. Georg Lamprecht and Ursula Seidler at the University of Tubingen, Germany. The full-length cDNA sequences of the SLC26A6 and SLC26A6del in the pcDNA3.1/v5/his-TOPO plasmid were digested by HindIII and EcoRV and subcloned into pinPoint (Promega); these constructs were called Biotin-26A6-C-terminus and Biotin-26A6del-C-terminus. To express biotinylated fusion proteins, an overnight culture (pinPoint construct in E.coli NM522) was diluted 1:100 in LB plus 50 μM Biotin and after 2.5 h 0.8 mM IPTG was added. After another 4.5 h, bacteria were spun down and sonicated in 100 mM NaCl, 100 mM Na_2HPO_4, pH 7.4 in a Branson sonifier. The material was finally spun at 20,000 g for 20 min and the supernatant saved. His-tagged fusion proteins of full length NHERF (amino acids 1-367) and E3KARP (1-337) as well as their individual PDZ domains (NHERF-PDZ1: 1-153, NHERF-PDZ2: 159-346, E3KARP-PDZ1: 1-141, E3KARP-PDZ2: 130-311) and their C-termini (NHERF: 266-368, E3KARP: 232-337) were expressed in pET30 (Novagen), affinity-purified under non-denaturing conditions using Nickel-nitrilotriacetic acid (NTA) resin as suggested by the manufacturer (QIAGEN Inc.) and finally eluted in 1 M imidazol, 150 mM NaCl, 10 mM Na_2HPO_4, pH 8. The His-tag fusion protein constructs of NHERF and E3KARP (about 4 μg) were diluted in siliconized tubes in 1 ml of interaction buffer (200 mM NaCl, 100 mM NaH_2PO_4, pH 7.5) to decrease the imidazol to less than 15 mM. 1 μl (20 μl of 5% suspension supplied by the manufacturer) of magnetic NTA-Ni agarose beads (Qiagen) was added. After 1 h the beads were separated using a magnet and the supernatant was removed. The beads were blocked for 10 min using 2% BSA in interaction buffer. 500 μl of the cleared bacterial lysate of Biotin-SLC26A6-C-terminus and Biotin-26A6del-C-terminus and 500 μl of 4% BSA (in interaction buffer) were added and the suspension was incubated for 4 h. The beads were washed 4 times with interaction buffer using the magnetic separator. Finally the bound material was eluted in 50 μl Laemmli sample buffer and separated on PAGE in the presence of SDS. After transfer, the nitrocellulose membranes were blocked with 3% BSA/TBS, and biotinylated proteins were detected using HRP-labeled Streptavidin and an ECL-detection system.

4.14. Real-time quantitative PCR (IV)

Real-time quantitative PCR can be used for sensitive measurement of different RNA levels. In this relatively new quantitative PCR method, gene specific probes labeled with fluorescent dyes are cleaved by 5´-3´-endonuclease activity of Taq DNA polymerase (Taqman universal PCR master mix, Perkin-Elmer). Sequence-specific signals from the fluorogenic probes are generated during the PCR. The fluorescence emission is gathered via optic cables and CCD camera into the computer. The Sequence Detection System software analyzes the data by first calculating the contribution of each component dye to the experimental spectrum. The reporter signal (FAM, JOE or VIC) is then normalized to the fluorescence of an internal reference dye (ROX). The parameter Ct (threshold cycle) is defined as the fractional cycle number at which the reporter fluorescence generated by cleavage of the probe passes a fixed threshold above baseline. The sensitive fluorescence detection allows the threshold cycle to be observed when PCR amplification is still in the exponential phase, rather than the amount of PCR product accumulated after a fixed number of cycles. Measuring Ct and comparing it to the Ct of the control gene accomplish relative quantitation of the amount of target in unknown samples. Adjacent colonic tissue samples were used for histological grading and RNA extraction. Total RNA was extracted from fresh tissues using RNeasy Mini Kit (Qiagen) and 400 ng total RNA was further transcribed to cDNA with random hexamers (Taqman Reverse Transcription Reagents, Perkin-Elmer). Dilutions of the cDNA were used for real-time quantitative PCR (5´ fluorogenic nuclease assay) using Perkin-Elmer´s ABI Prism 7700 Sequence Detector System (23). The probes used were CCA ATC GAA TTC ATT ATG ACC GTG ATT GC for SLC26A3, ATG CAG TCT CTG GAG CAG CGA CGG for SLC9A3 and CAG AAG CGT CAT CAA AGC ATG CCA AC for CFTR. Primers were designed into exon-intron junctions to avoid amplification of genomic DNA. A S18 rRNA probe and primers were used as an endogenous control gene (S18 control kit, Perkin-Elmer). PCR conditions were: 2 min 50°C, 10 min 95°C and 40 cycles of 15 sec 95°C, 1 min 60°C. The PCR assays were performed in separate tubes and relative quantitation of the SLC26A3, SLC9A3 and CFTR mRNAs was performed using the standard curve method according to the manufacturer's instructions (PE Applied Biosystems, User Bulletin #2: ABI PRISM 7700 Sequence Detection System).

4.15. Tissue samples and histological grading (IV)

Altogether 30 peroperative tissue samples from 10 patients undergoing surgery for ulcerative colitis and 12 samples from 4 control individuals with non-inflamed colon mucosa were obtained from the Department of Surgery, Helsinki University Central Hospital by permission of the respective

Ethical Review Board. Inflamed colonic tissue samples were taken from two areas: those that macroscopically appeared normal and those demonstrating the highest degree of inflammation. Control tissue samples were taken from morphologically unaltered, uninflamed colonic mucosa of benign (tubular adenoma) or malignant (two adenocarcinomas) tumors at distant nonneoplastic area, as well as of diverticulosis. Tissue samples used for mRNA and protein analysis were taken from the same area as those samples used for histopathological analysis. Tissue samples were handled immediately after surgery on ice or snap frozen in liquid nitrogen before homogenization. The samples for histological analyses were fixed in 10% neutral buffered formalin immediately after surgery. Formalin-fixed paraffin-embedded samples were prepared for histology and stained by hematoxylin-eosin for histological grading. The degree of inflammation was graded (Truelove and Richards 1956) on a four point scale; normal, (no significant inflammation, n=12); mild, (elevated number of mucosal leukocytes but intact epithelium, n=11); moderate, (aggregates of leucocytes with crypt abscesses and erosions but no ulceration of the epithelium, n=7); and severe (significant ulceration of the epithelium by mononuclear cell infiltrate, n=12). Histological grading was performed by a pathologist (Marja-Liisa Karjalainen-Lindberg, Dept. of Pathology) without knowledge of surgery or the endoscopic reports and experimental data.

4.16. Immunoblotting (IV)

Mucosal scrapings of human colon tissues were transferred to ice-cold lysis buffer (50 mM core buffer (Roche), 150 mM NaCl, 0,1 mg/ml phenylmethylsulfonyl fluoride, 1 µg/ml aprotin, 1 µg/ml leupeptin, 1% Nonidet P-40, 0,5% sodium deoxycholate). The lysed cells were homogenized by a syringe and needle followed by centrifugation at 12000 g at 4°C for 10 minutes. The supernatant was preserved for protein analyses. Protein concentration was measured using the colorimetric Bradford protein assay (Bio-Rad Lab., Hercules, California, USA). Total proteins were separated by 9% SDS-PAGE, and electroblotted onto Hybond C-extra membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). Non-specific binding sites were saturated by incubating the membranes in a solution of 5% non-fat dry milk in PBS containing 0.1% of Tween-20. Appropriate dilutions of the primary antibodies were determined by immunoblotting, and biotin conjugated goat anti-rabbit IgG (Roche, Basel, Switzerland) was used as a secondary antibody in PBS. To visualize the protein bands by enhanced chemiluminescent reaction, membranes were incubated in dilution of horseradish peroxidase conjugated streptavidin (Roche). Computer assisted scanning densitometry (Biometra, BioDocAnalyze, Göttingen, Germany) was used to analyze the intensity of the immunoreactive bands in the autographs. The optic densities were normalized to the amount of

protein in lane. Statistical significance between groups was analyzed by the nonparametric Kruskal-Wallis test.

4.17. Statistical analysis (IV)

Statistical analyses were performed using the PRISM Statistic Package, version 3.0 (GraphPad Software Inc. San Diego, California, USA). The degree of statistical significance between two groups was calculated using the nonparametric Mann-Whitney U-test. Comparisons between three or more groups were performed using the Kruskal-Wallis test. The groups were considered different at a p value <0.05. In these cases, p values for comparisons between groups were calculated using Dunn´s multiple comparison as a nonparametric post-test.

5. RESULTS

5.1. Identification of seven novel human SLC26 members with distinct map positions and expression patterns (I-II)

A homology approach using the public databases mainly through the NCBI BLAST server was utilized in a search for new members of the SLC26 family; much of the initial database work was done in 1998-1999, and only using publicly available sequences. Protein and nucleotide sequence homology searches with the three known human sequences SLC26A2-A4 (U14528, L02785 and AF030880, respectively) revealed several significant matches against ESTs and genomic sequences (BLASTX E-value >1e-20). The characterization of these new candidates was initiated by assigning the chromosomal locations using radiation hybrid panels and found matches to genomic clones deposited in GenBank. The coding regions of some candidates were expanded by sequencing the corresponding IMAGE clones. In addition, the preliminary tissue distribution was determined using Clontech´s multiple tissue cDNA panels.

Altogether, seven novel loci with tissue specific expression patterns were established including the human ortholog of rat slc26a1 gene (Table 2). Subsequently, five of the candidates were cloned and characterized.

Table 2. Summary of the new candidate members of the SLC26 family found by homology searches.

Candidate sequences	Length of the translated sequence	Degree of amino acid homology	Chromosomal location	Major site of expression
EST AW001016	130 aa	84% to rat slc26a1	4p16.3	Liver, kidney, pancreas, brain
EST AA411587	120 aa	~50% to SLC26A3	3p21.3	Kidney, pancreas
genomic PAC 179N16	~300 aa predicted	~50% to SLC26A3	6p21.1	Testis
EST AA992584	376 aa	48% to SLC26A2	8q23	Kidney, testis, placenta
unspliced cDNA AL050358	427 aa	~60% to SLC26A4	12q13	Brain, other?
EST AA348883	300 aa	Homologous to insect and plant orthologs	17q25	Ubiquitous
genomic PAC clone RP11-370I5 (AL360009)	~500 aa predicted	A match of E-value=0.001 with SLC26A3	1q32	Lung

5.2. Characteristics of the novel family members (I-III)

After the mapping and identification of the new candidates, further studies concentrated on the characterization of the structure, expression and function of the SLC26A6-A9 genes. In addition, the human ortholog of rat slc26a1 was mapped and cloned. Sequences have been submitted to GenBank and the nomenclature has been verified with the HUGO Nomenclature Committee. Subsequently, the new human genes have been designated SLC6A1 and SLC26A6-A11. The characterization of SLC26A10 and A11 is still poorly known and continues.

SLC26A1. Homology screenings found a human EST sequence (AW001016), which showed the highest homology (84%) to rat sulfate/anion exchanger, suggesting a putative ortholog for rat slc26a1. The identified EST sequence matched to a genomic sequence recently emerged in GenBank facilitating the cloning of the human SLC26A1 sequence (GenBank AF297659). Sequence analysis revealed that the SLC26A1 cDNA (~3.6kb) contains three exons and translates into a protein of 701 amino acids, which shares 77% sequence identity with rat slc26a1. The murine slc26a1 gene was also characterized recently (Xie et al. 2002). Its predicted protein is 91% and 76% identical to the rat and human SLC26A1 proteins, respectively. SLC26A1 shows the best sequence similarity to SLC26A2 within the SLC26 family (Fig. 8). The gene maps to 4p16.3 and locates within another gene on the opposite strand encoding the enzyme α-L-iduronidase (IDUA). Initial expression analysis showed abundant expression in the liver, kidney, pancreas and brain, resembling that of the rat slc26a1 (Bissig et al. 1994; Markovich et al. 1994; Karniski et al. 1998). Thus SLC26A1 is likely to be the human counterpart of the rat slc26a1 gene because of its high sequence homology, resembling expression pattern and corresponding syntenic chromosome region with the rat and mouse slc26a1 genes.

SLC26A6. The cloning of the putative new gene (EST AA411587) on 3p21.3 was accomplished by verifying the predicted exons in the human genomic clone. Sequence analysis revealed an open reading frame of 2217 bp encoding a 738 amino acid protein, which has been designated SLC26A6 (GenBank AF279265). The open reading frame of SLC26A6 is distributed across 20 exons ranging in size from 55 to 177 bp. Exon-intron boundaries obey the general AG-GT rule except for exon 15 that ends to GC. Interestingly, 12 of 20 exons are exactly of the same size as those of the SLC26A3 and SLC26A4 genes, which share 15 exons of similar size with each other. The putative ATG translation start site is within the Kozak consensus sequence (Kozak 1987), and four in-frame stop codons precede that methionine. A 280-bp 3' untranslated region precedes the consensus polyadenylation signal AATAAA.

The SLC26A6 protein shows the best sequence similarity (56%) to SLC26A5 with a suggested function as a cochlear motor protein (Fig. 8). Twelve-transmembrane structure with intracellular N- and C-terminal domains, suggested by the PSIpred program, resembles the topology predicted for the human SLC26A2 and SLC26A3 proteins (Kere et al. 1999). ProfileScan analysis of the SLC26A6 protein sequence revealed two domains, which are also commonly shared within the other SLC26 family members (Table 4): Sulfate Transporter family domain (ST, predicted between aa ~200-500, PF00916) and Sulfate transporters and Antisigma-factor antagonists domain (STAS, between aa ~500-700, PS50801). The putative NTP-binding STAS domain suggests that anion transport could be regulated by intracellular nucleotides (Aravind and Koonin 2000). In addition, C-terminus of the SLC26A6 protein (TRL) comprises the consensus PDZ interacting motif T/S-X-Φ, in which Φ is a hydrophobic amino acid (Songyang et al. 1997). The PDZ domain proteins play an essential role in maintaining the cell polarity and function (Caplan 1997; Aroeti et al. 1998; Fanning and Anderson 1999).

Soon after the publication of the SLC26A6 gene, it was cloned also by another group (Waldegger et al. 2001). However, their sequence differs in 5´ noncoding and coding region leading to an addition of 21 amino acids to the ultimate N-terminus of the gene. The sequence flanking the putative translation start site contained a Kozak sequence. It is possible that this represents an alternative N-terminal variant of SLC26A6, because our 5´noncoding region contained four in-frame stop codons, and also our putative translation initiation site resided within a Kozak consensus sequence.

Similarly, two alternative 5´noncoding regions and translation sites with Kozak sequences were identified in the mouse slc26a6 gene (Xie et al. 2002). Inclusion of the exon 1b in the longer slc26a6b transcripts results in a frameshift and a start codon within exon 2. The predicted slc26a6b protein is thus 23 amino acids shorter than slc26a6a. The starts codons in exon 1a and 2 are both predicted Kozak sites. Slc26a6b is identical to the sequence of mouse chloride-formate exchanger, CFEX (Knauf et al. 2001), and corresponds to our SLC26A6, whereas the longer slc26a6a sequence corresponds to SLC26A6 reported by Waldegger et al. The murine slc26a6 and human SLC26A6 orthologs have similar genomic structures, but their share only 78% identity at the amino acid level (Xie et al. 2002).

During the cloning of the full-length cDNA sequence of SLC26A6 (AF279265), several transcripts were repetitively obtained from RT-PCR amplifications and Northern blots, suggesting the presence

of alternatively spliced forms of the SLC26A6 gene. We cloned and characterized two of them, named SLC26A6c and SLC26A6d. For clone nomenclature, the isoforms were denoted SLC26A6a-A6d (A6b cloned by Waldegger et al. 2001) to portray the order of reporting these isoforms. The presence of the SLC26A6a, SLC26A6c and SLC26A6d transcripts in human kidney and lung total RNA or mRNA were verified by ribonuclease protection assays.

Sequence analysis of SLC26A6c and SLC26A6d revealed an open reading frame of 2100 bp and 2016 bp encoding 699 and 671-amino-acid proteins, respectively (Fig. 7B). The topology of SLC26A6a, SLC26A6c and SLC26A6d analysed by PSIpred 2.0 method and hidden Markov model (TMHMM program) resulted in the prediction of 12-, 8- and 12-transmembrane and 10-, 8- and 10-transmembrane helices with intracytoplasmic NH_2 and COOH termini, respectively.

Comparison of the nucleotide sequences of SLC26A6a and SLC26A6c revealed a 114 bp deletion within exon 6 and a 3 bp deletion in the beginning of the exon 17 produced by alternative usage of splice donor (GT) and acceptor sites, respectively. This results in a deletion of amino acids 243 to 281 and a glutamine residue (Q) at position 611 compared to SLC26A6a. Thus the single residue polymorphism at position 611 found previously appeared to be a part of the SLC26A6c isoform (Fig. 7A). Deletion of the 38 residues results in the loss of two transmembrane domains (TMD #5 and #6) and predicts an additional large extracellular domain after TMD #4. A similar change has been described previously in the analysis of alternative splicing of rat liver-specific organic anion transporter-1 (Choudhuri et al. 2000).

The SLC26A6d sequence retains an unspliced intron, intron 16 (892 bp), which includes several stop codons (Fig. 7A). This leads to an alternative C-terminus of the protein compared with SLC26A6a, which may affect the regulation or function of SLC26A6d. Specifically, the C-terminus of SLC26A6d lacks the PDZ interaction motif, which is included in SLC26A6a and SLC26A6c.

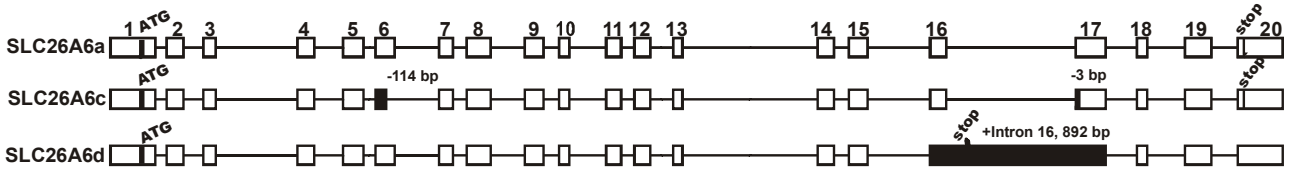

Figure 7A. Genomic structures of the SLC26A6 isoforms.

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SLC26A6a    1 MDLRRRDYHMERPLLNQEHLEELGRWGSAPRTHQWRTWLQCSRARAYALLLQHLPVLVWL
SLC26A6c    1 MDLRRRDYHMERPLLNQEHLEELGRWGSAPRTHQWRTWLQCSRARAYALLLQHLPVLVWL
SLC26A6d    1 MDLRRRDYHMERPLLNQEHLEELGRWGSAPRTHQWRTWLQCSRARAYALLLQHLPVLVWL

SLC26A6a   61 PRYPVRDWLLGDLLSGLSVAIMQLPQGLAYALLAGLPPVFGLYSSFYPVFIYFLFGTSRH
SLC26A6c   61 PRYPVRDWLLGDLLSGLSVAIMQLPQGLAYALLAGLPPVFGLYSSFYPVFIYFLFGTSRH
SLC26A6d   61 PRYPVRDWLLGDLLSGLSVAIMQLPQGLAYALLAGLPPVFGLYSSFYPVFIYFLFGTSRH

SLC26A6a  121 ISVGTFAVMSVMVGSVTESLAPQALNDSMINETARDAARVQVASTLSVLVGLFQVGLGLI
SLC26A6c  121 ISVGTFAVMSVMVGSVTESLAPQALNDSMINETARDAARVQVASTLSVLVGLFQVGLGLI
SLC26A6d  121 ISVGTFAVMSVMVGSVTESLAPQALNDSMINETARDAARVQVASTLSVLVGLFQVGLGLI

SLC26A6a  181 HFGFVVTYLSEPLVRGYTTAAAVQVFVSQLKYVFGLHLSSHSGPLSLIYTVLEVCWKLPQ
SLC26A6c  181 HFGFVVTYLSEPLVRGYTTAAAVQVFVSQLKYVFGLHLSSHSGPLSLIYTVLEVCWKLPQ
SLC26A6d  181 HFGFVVTYLSEPLVRGYTTAAAVQVFVSQLKYVFGLHLSSHSGPLSLIYTVLEVCWKLPQ

SLC26A6a  241 SKVGTVVTAAVAGVVLVVVKLLNDKLQQQLPMPIPGELLTLIGATGISYGMGLKHRFEVD
SLC26A6c  241 SK-----------------------------------LIGATGISYGMGLKHRFEVD
SLC26A6d  241 SKVGTVVTAAVAGVVLVVVKLLNDKLQQQLPMPIPGELLTLIGATGISYGMGLKHRFEVD

SLC26A6a  301 VVGNIPAGLVPPVAPNTQLFSKLVGSAFTIAVVGFAIAISLGKIFALRHGYRVDSNQELV
SLC26A6c  263 VVGNIPAGLVPPVAPNTQLFSKLVGSAFTIAVVGFAIAISLGKIFALRHGYRVDSNQELV
SLC26A6d  301 VVGNIPAGLVPPVAPNTQLFSKLVGSAFTIAVVGFAIAISLGKIFALRHGYRVDSNQELV

SLC26A6a  361 ALGLSNLIGGIFQCFPVSCSMSRSLVQESTGGNSQVAGAISSLFILLIIVKLGELFHDLP
SLC26A6c  323 ALGLSNLIGGIFQCFPVSCSMSRSLVQESTGGNSQVAGAISSLFILLIIVKLGELFHDLP
SLC26A6d  361 ALGLSNLIGGIFQCFPVSCSMSRSLVQESTGGNSQVAGAISSLFILLIIVKLGELFHDLP

SLC26A6a  421 KAVLAAIIIVNLKGMLRQLSDMRSLWKANRADLLIWLVTFTATILLNLDLGLVVAVIFSL
SLC26A6c  383 KAVLAAIIIVNLKGMLRQLSDMRSLWKANRADLLIWLVTFTATILLNLDLGLVVAVIFSL
SLC26A6d  421 KAVLAAIIIVNLKGMLRQLSDMRSLWKANRADLLIWLVTFTATILLNLDLGLVVAVIFSL

SLC26A6a  481 LLVVVRTQMPHYSVLGQVPDTDIYRDVAEYSEAKEVRGVKVFRSSATVYFANAEFYSDAL
SLC26A6c  443 LLVVVRTQMPHYSVLGQVPDTDIYRDVAEYSEAKEVRGVKVFRSSATVYFANAEFYSDAL
SLC26A6d  481 LLVVVRTQMPHYSVLGQVPDTDIYRDVAEYSEAKEVRGVKVFRSSATVYFANAEFYSDAL

SLC26A6a  541 KQRCGVDVDFLISQKKKLLKKQEQLKLKQLQKEEKLRKQAASPKGASVSINVNTSLEDMR
SLC26A6c  503 KQRCGVDVDFLISQKKKLLKKQEQLKLKQLQKEEKLRKQAASPKGASVSINVNTSLEDMR
SLC26A6d  541 KQRCGVDVDFLISQKKKLLKKQEQLKLKQLQKEEKLRKQAASPKGASVSINVNTSLEDMR

SLC26A6a  601 SNNVEDCKMMQVSSGDKMEDATANGQEDSKAPDGSTLKALGLPQPDFHSLILDLGALSFV
SLC26A6c  563 SNNVEDCKMM-VSSGDKMEDATANGQEDSKAPDGSTLKALGLPQPDFHSLILDLGALSFV
SLC26A6d  601 SNNVEDCKMM------------------VRLEVGKEVTAVSCRDAGSTCLMRNAMDPAAV

SLC26A6a  661 DTVCLKSLKNIFHDFREIEVEVYMAACHSPVVSQLEAGHFFDASITKKHLFASVHDAVTF
SLC26A6c  622 DTVCLKSLKNIFHDFREIEVEVYMAACHSPVVSQLEAGHFFDASITKKHLFASVHDAVTF
SLC26A6d  643 GSRVLRRWQEEWGGW-------------------------------------------

SLC26A6a  721 ALQHPRPVPDSPVSVTRL
SLC26A6c  682 ALQHPRPVPDSPVSVTRL
SLC26A6d  658 ----VRYSSGSVVICHRI
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Figure 7B. Comparison of the amino acid sequences of the SLC26A6 variants. Predicted transmembrane segments are shadowed by grey.

SLC26A7. The SLC26-related EST AA992584 mapped to chromosome 8 was expanded and its sequence revealed an open reading frame of 1971 bp encoding a 656 amino acid protein designated SLC26A7 (GenBank Accession No. AF331521). The SLC26A7 gene spans about 100 kb of genomic sequence and the open reading frame is distributed across 19 exons ranging in size from 55 to 306 bp. A ~5250-bp cDNA of SLC26A7 includes four in-frame stop codons upstream the initiation methionine and a 3024-bp 3' untranslated region before the polyadenylation signal AATAAA. Moreover, several alternative polyA signals were identified at the 3´end of the SLC26A7 composite sequence, suggesting alternative polyadenylation of the gene.

Consistent with this notion, the SLC26A7 gene was cloned from human high endothelial venule cells (HEVEC) (Vincourt et al. 2002). Alternative polyadenylation resulted in a shorter 2.9-kb cDNA, which encoded an identical 656-amino acid protein. Furthermore, a C-terminal kidney-specific variant, SLC26A7.2, was reported. The last 11 residues of SLC26A7 are replaced by a novel 18-amino acid sequence in the 663-amino-acid SLC26A7.2 isoform (Vincourt et al. 2002).

Table 3. The exon structure and conservation within the SLC26 family

	1	2	**3**	**4**	5	**6**	**7**	**8**	**9**	**10**	**11**
SLC26A3	107	219	**140**	**111**	188	**165**	**153**	**83**	**148**	**114**	**78**
SLC26A4	221	167	**140**	**111**	185	**165**	**153**	**83**	**148**	**114**	**78**
SLC26A6		159	**140**	**111**	152	**165**	**153**	**83**	**148**	**114**	**78**
SLC26A7		96	306	**111**	173	**165**	**153**	**83**	**148**	**114**	**78**
SLC26A8	110	190	**140**	116	162	**165**	**150**	**83**	**148**	**114**	**78**
SLC26A9	96	142	**140**	**111**	176	**165**	**153**	**83**	**148**	**114**	**78**

	12	**13**	**14**	**15**	16	17	18	19	20	21
SLC26A3	**96**	**107**	**70**	**93**	**96**	234	**55**	**146**	67	21
SLC26A4	**96**	**107**	**70**	**93**	**96**	231	**55**	**146**	84	23
SLC26A6	**96**	**107**	**70**	**93**	118	93	177	55	137	15
SLC26A7	**96**	**107**	67	138	49	101	**55**	104	36	
SLC26A8	**96**	**107**	**70**	**93**	132	369	**55**	185	441	
SLC26A9	**96**	**107**	**70**	**93**	114	282	**55**	**146**	72	48

The SLC26A7 protein shows ~50% similarity to SLC26A2 and A3 proteins (BLASTP E-value ~1e-80). Two putative N-glycosylation sites at Asn^{125} and Asn^{131} were identified. Furthermore, the SLC26A7 protein sequence has ST and STAS domains and the C-terminus (SEV) comprises the consensus PDZ interacting motif (Table 4). PSIpred program predicted a 12-transmembrane structure with intracellular N- and C-terminal domains.

SLC26A8. The complete sequence of SLC26A8 was constructed from two different genomic clones in chromosome 6. In addition, the 5´and 3´-regions were expanded and verified by RT-PCR. The complete cDNA sequence (GenBank Accession No. AF331522) includes an open reading frame of 1971 bp encoding a 970 amino acid protein. The SLC26A8 gene contains 20 exons and spans about 80 kb of genomic sequence. The putative ATG translation start site is within the Kozak consensus sequence and a 361-bp 3' untranslated region precedes the consensus polyadenylation signal AATAAA. A number of exons show conservation of the size, although there are more variations when compared with the other family members.

The SLC26A8 protein shows the best sequence similarity to SLC26A3 and SLC26A6 (BLASTP E-value= 2e-78 and 1e-73, respectively). However, the SLC26A8 protein possesses 200-300 amino acids more than the other members and SLC26A8-specific extra sequences were found between amino acids 600-652 and C-terminal regions. Furthermore, comparison of the SLC26A8 protein with the other family members reveals several unconserved residues (Fig. 8). Altogether eight putative N-glycosylation sites were found along the protein at Asn^{52}, Asn^{192}, Asn^{277}, Asn^{384}, Asn^{595}, Asn^{651}, Asn^{687} and Asn^{688}. The eleven-transmembrane structure of the SLC26A8 protein with intracellular N-terminal domain and extracellular C-terminal domain was predicted by TMHMM program, whereas PSIpred failed to predict any structures for the protein. ProfileScan analysis of the SLC26A8 protein sequence found ST (aa 212-521) and STAS (aa 544-791) domains (Table 4).

Table 4. Analysis of the common conserved domains of the SLC26 family by ProfileScan.

Domain	Profile	A1	A2	A3	A4	A5	A6	A7	A8	A9
ST FAMILY	PF00916	36.5	39.5	37.8	33.5	33.5	34.9	29	27.1	29.4
STAS	PS50801	18.4	18.1	14.7	18.8	16.7	10.9	17.6	11.6	17.4
ST SIGNAT	PS01130	1.0	1.0	1.0	1.0	-	-	-	-	-
NLS BP	PS50079	-	-	3.0	-	3.0	-	-	3.0	-
XAN UR PERM	PF00860	-	-	8.5	8.6	-	8.5	9.2	9.5	-
PROK LIPOPRO	PS00013	1.0	-	1.0	1.0	1.0	-	1.0	1.0	1.0
PDZ	T/S-X-ϕ	-	X	X	-	-	X	X	-	X

ST FAMILY, sulfate transporter family domain; STAS, sulfate transporters and antisigmafactor antagonists; ST SIGNATURE, sulfate transporter signature; NLS BP, nuclear localization bipartite signal; XAN UR PERM, xanthine/uracil permeases family; PROK LIPOPRO, prokaryotic membrane lipoprotein lipid attachment site; PDZ, PSD-95/Disc-large/ZO-1 domain; ϕ is a hydrophobic amino acid

SLC26A9. A homology search with the sulfate transport signature motif of the human SLC26A3 protein against NCBI´s htgs database with TBLASTN algorithm resulted in an interesting match (E-value=0.001) in the genomic PAC clone RP11-370I5 (GenBank accession AL360009) in chromosome 1. Subsequent cloning efforts revealed a cDNA sequence including an open reading frame of 2373 bp encoding a 791 amino acid protein designated SLC26A9 (GenBank Accession AF331525).

The total length of the gene spans about 25 kb of the genomic sequence. The open reading frame is distributed across 21 exons ranging in size from 55 to 282 bp. Exon-intron boundaries obey the general AG-GT rule and 14 of 20 exons are exactly of the same size as those of the SLC26A3 and SLC26A4 genes. A 115-bp untranslated region including the first exon precedes that methionine and a putative TATA box lies 25 bp upstream from the first exon. A 2308-bp 3' untranslated region precedes the consensus polyadenylation signal AATAAA. Futhermore, PCR analysis proposed two alternatively spliced variants for SLC26A9. Sequencing revealed that the second or fourth exon of SLC26A9 is spliced out, which results in the truncation of the first 89 N-terminal amino acids or the deletion of amino acids 90 to 126 (37 residues) of the protein, respectively.

The SLC26A9 protein is highly conserved with the known family member (Fig. 8). SLC26A9 has two putative N-glycosylation sites at Asn^{153} and Asn^{156}. ProfileScan analysis of the SLC26A9 protein sequence revealed ST (aa 187-497) and STAS (aa 520-733) domains (Table 4). The C-terminus of the SLC26A9 protein (TAL) comprises the consensus PDZ-protein interacting motif (Table 4). The nine-transmembrane structure of the protein with intracellular N-terminal and extracellular C-terminal domains was suggested by the PSIpred and TMHMM programs.

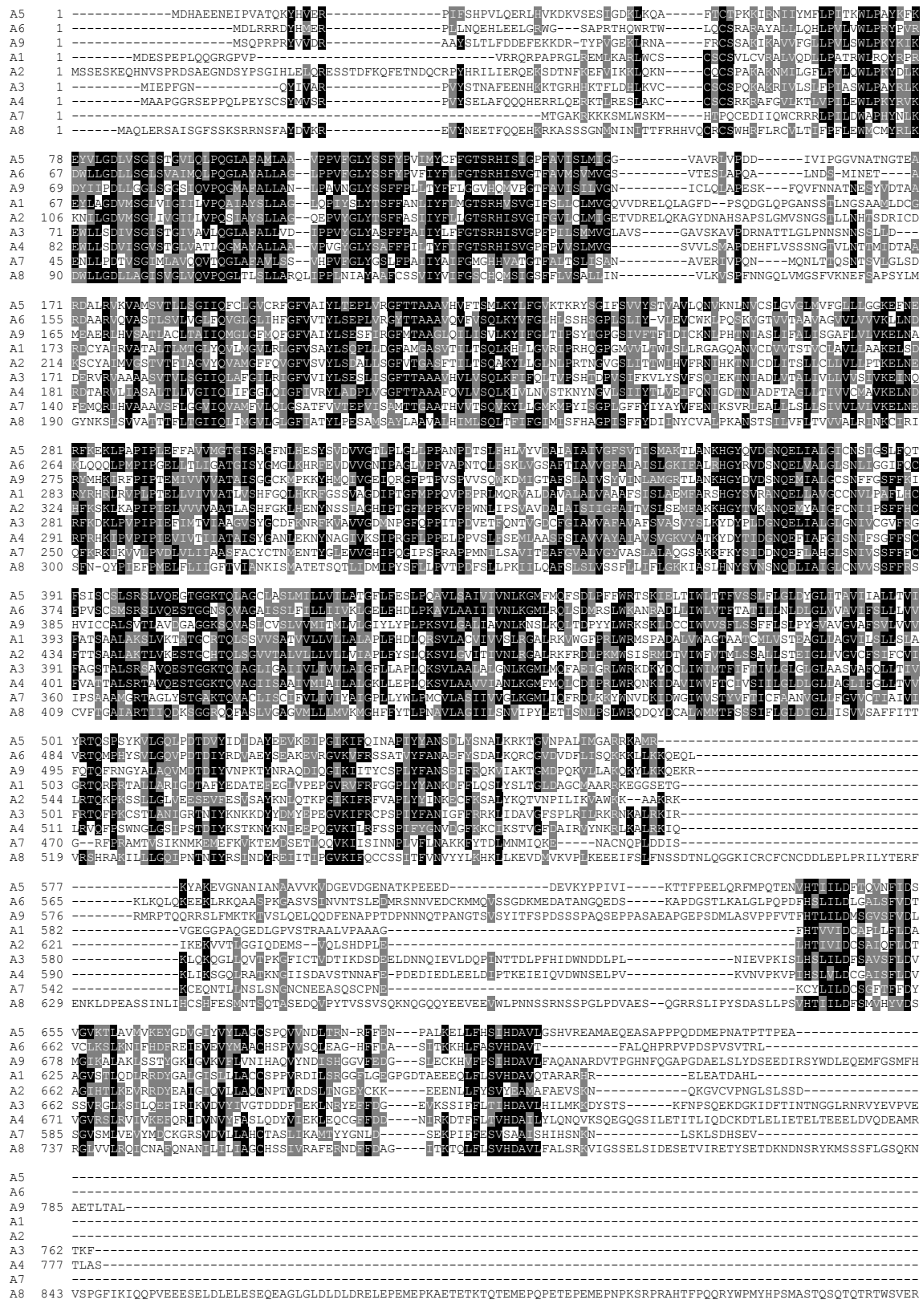

Figure 8. Multiple alignment of the SLC26 family members.

5.3. Tissue-specific expression patterns of SLC26A6-A9

SLC26A6. The 2748 bp full-length cDNA of SLC26A6a corresponds well with the observed size (~3 kb) in northern analysis, and it revealed a specific tissue distribution, with most abundant expression in kidney, pancreas, placenta and skeletal muscle. PCR analysis demonstrated that SLC26A6a is widely expressed, but at highest level in kidney and pancreas. PCR analysis suggested also the existence of alternatively spliced forms of the gene, in accordance with the northern analysis.

To verify and refine the expression results, we analysed two human pancreatic duct cell lines, Capan-1 and Capan-2, and the human embryonal kidney (HEK) cell line 293 by RT-PCR. All three cell lines expressed SLC26A6. Characterization by in situ hybridization and immunohistochemistry detected SLC26A6 at apical and basolateral surface of tubular walls in kidney and in the brush border of pancreatic duct, suggesting a functional role for SLC26A6 as a pancreatic and/or renal anion exchanger.

Tissue distribution of the SLC26A6a, SLC26A6c and SLC26A6d variants were compared by PCR amplification of multiple tissue cDNA panels. Specific primers were designed to amplify both SLC26A6a and A6c genes at the same reaction. SLC26A6a was detected in all tissues, while SLC26A6c showed a more restricted expression pattern. The expression of SLC26A6d gene was observed in many tissues, but mainly in the kidney and pancreas. All isoforms were expressed in the pancreatic ductal cell line, Capan-1. These results suggested tissue-specific functions for different SLC26A6 variants.

The exact topologies of the SLC26 anion exchangers are uncertain. In order to determine the location of the N- and C-termini of the SLC26A6 variants relative to plasma membrane, anti-SLC26A6 antibodies specific to the N- and C-terminal tails were used in permeabilized and nonpermeabilized SLC26A6a, A6c and A6d-transfected cells. Transiently transfected COS-1 cells expressed all isoforms as shown with permeabilized cells, while untransfected cells remained negative. However, no labelling was observed either with N-terminal or C-terminal antibodies in non-permeabilized cells with SLC26A6a and A6c isoforms, demonstrating that both the N- and C-termini of the proteins are located intracellularly. Similarly, the N-terminus of SLC26A6d was located intracellularly. In the lack of a specific antibody the altered C-terminus of SLC26A6d compared to SLC26A6a could not be localized. Similar results were observed also with an alternative permeabilization procedure with saponin (unpublished results).

The specificity of the antibodies was confirmed by western blotting, which showed that both N- and C-terminal antibodies bind specifically to the SLC26A6a protein (about 85 kDa) in transfected cells as compared with the untransfected cells. Similar results were observed also with in vitro translated SLC26A6a protein. The membranes stained with preimmune sera remained negative. Altogether, our results support models with an even number of transmembrane helices, consistent with the predicted topology of SLC26A6a, SLC26A6c and SLC26A6d proteins. Thus, the topologies of the SLC26A6 variants resemble the model suggested for SLC26A5, which is the closest homolog of SLC26A6. It follows from these results that the N- and C- termini are possible sites for interaction with cytoplasmic proteins.

SLC26A7. The tissue distribution of the SLC26A7 gene was determined by northern hybridization and PCR using Clontech´s multiple tissue cDNA panels with 16 different tissues. The 5250 bp full-length cDNA of SLC26A7 corresponds well with the observed size (~5 kb) in northern analysis, and it revealed a specific tissue distribution, with most abundant expression in the kidney. A weaker ~3.0 kb transcript was also observed and it might be derived from an alternative polyadenylation of the gene. The long 3´UTR of the SLC26A7 gene contains a number of consensus AAUAAA or AUUAAA sequences that may serve as alternative polyadenylation signals in a reasonable agreement with the size of the less abundant ~3.0 kb transcript. PCR analysis showed also most abundant expression in kidney, placenta and testis supporting the northern analysis. Analysis of SLC26A7 expression by immunohistochemistry localized it to distal segments of nephrons in the kidney, while the proximal tubules remained negative.

SLC26A8. Both northern and PCR analyses revealed a testis-specific distribution for SLC26A8. The 3371 bp cDNA of SLC26A8 corresponded with the observed size (~3.5 kb) in northern analysis. Cell-specific localization of SLC26A8 using in situ hybridization and immunohistochemistry suggest that the expression of SLC26A8 is restricted to the meiotic phase of the germ cells in the human testis. The SLC26A8 antisense cRNA probe revealed abundant expression in the seminiferous tubules and mRNA was concentrated on the luminal side of the tubuli harboring the spermatocytes and the spermatids, while the peripheral side of the tubuli, containing the spermatogonia, appeared negative. Consistently, immunohistochemical stainings localized SLC26A8 protein exclusively to developing spermatocytes and spermatids.

SLC26A9. PCR analysis suggested that SLC26A9 is expressed predominantly in the lung, although some expression was found also in the pancreas, prostate and kidney. The lung-specific expression of SLC26A9 was confirmed by northern blotting. The 4815 bp cDNA of SLC26A9 corresponded with the observed size (~4.8 kb) in northern analysis. SLC26A9 expression was found also in two human lung-derived carcinoma cell lines, NCI-H358 and A549, from bronchoalveolus and alveolus, respectively. Furthermore, SLC26A9 was detected in the bronchiolar and alveolar epithelium of lung by immunohistochemistry.

5.4. SLC26A6-A9 function as anion exchangers and may interact with PDZ proteins

The high homology of the SLC26A6-A9 proteins to the known family members suggested a related transport functions. Functional characterization of the proteins was performed in *Xenopus laevis* oocytes. In vitro transcribed cRNAs were injected into oocytes and transport function was measured by ^{35}S-sulfate, ^{36}Cl-chloride and ^{14}C-oxalate uptakes. Expression of SLC26A6 isoforms revealed induction of chloride and sulfate transport compared to water controls. Furthermore, the sulfate uptake was inhibited by anion exchanger inhibitor, DIDS, and also by HCO_3^-, suggesting that bicarbonate competes the same binding site of the proteins with sulfate and would be therefore a substrate as well. We found also that SLC26A6a accepts oxalate as a substrate.

Similarly, the expression of SLC26A7-A9 proteins led to the induction of chloride, sulfate and oxalate transport above water-injected oocytes. Furthermore, the SLC26A9-mediated sulfate transport was inhibited by DIDS and thiosulfate, but not by oxalate or glucose. Similar inhibition of SLC26A8 activity by DIDS was observed previously (Toure et al. 2001). During this study, the characterization of a novel sulfate transporter, Tat1 (testis anion transporter 1), was published (Toure et al. 2001). Our results identify Tat1 and SLC26A8 as the same gene and confirm observation regarding its expression and function. Altogether, these results demonstrate that SLC26A6-A9 proteins function as novel anion exchangers.

Recent studies have demonstrated that PDZ domain proteins play an essential role in the assembly of multiprotein complexes involved in determining cell polarity, plasma membrane targeting and regulation of membrane proteins (Fanning et al 1998). Since the ultimate C-terminus of SLC26A6a and SLC26A6c contain a consensus PDZ-interaction motif (TRL), it prompted us to test its putative binding to different PDZ domains of the E3KARP and NHERF proteins in vitro. The intact C-terminus of SLC26A6a and A6c, but not the SLC26A6adel mutation missing the PDZ interaction motif, bound to full length NHERF and E3KARP. This result demonstrated the functionality and

specificity of the C-terminal TRL motif of SLC26A6a and SLC26A6c for interaction with NHERF and E3KARP. In order to test whether this interaction indeed occurred through the PDZ domains of these proteins, the PDZ domains and the C-terminus of NHERF and E3KARP were individually tested for binding. SLC26A6 was found to bind to both PDZ domains but not to the C-terminus of NHERF and E3KARP. Interestingly, many other SLC26 family members possess also the consensus PDZ interaction motif in the C-termini, suggesting that the PDZ interaction pathways play an important role in the regulation of this group of transporters.

5.5. Upregulation of CFTR but not SLC26A3 and SLC9A3 expression in ulcerative colitis

In normal colonic mucosa, the absorption of salt is driven by active transporters, followed by passive movements of water. Much of the absorption of NaCl is mediated by electroneutral pH-coupled Na^+/H^+ and Cl^-/HCO^-_3 exchangers (Knickelbein et al. 1985). SLC26A3 most likely functions as a major Cl^-/HCO_3^- exchanger in the colon, and much of the intestinal Na^+/H^+ exchanger activity is mediated by SLC9A3 (known also as NHE3) (Kere et al. 1999; Gawenis et al. 2002). The latter notion is supported by the targeted disruption of the SLC9A3 gene, which results in sodium diarrhea and acidosis (Schultheis et al. 1998). The SLC9A3 gene belongs to the sodium proton exchanger (NHE) family, SLC9, in which seven different isoforms have been identified to date. Cystic fibrosis transmembrane regulator (CFTR), the gene for one of the most common inherited diseases worldwide, cystic fibrosis, acts as the major mediator of cAMP- and Ca^{2+}-activated Cl^- secretion in the colonic epithelium (Mall et al. 1998).

In inflamed colonic mucosa, the equilibrium between absorptive and secretory functions for electrolyte and water transport is disturbed. Therefore, we compared the expression of three major mediators of the intestinal salt transport, CFTR, SLC26A3 and SLC9A3 between healthy and inflamed colonic mucosa to understand the pathophysiology of diarrhoea in inflammatory bowel disease. Real-time quantitative RT-PCR, immunohistochemistry and Western blotting were applied to measure the expression levels in peroperative samples from normal (n=12) and UC patients´ colon (n=30). Expression levels were normalised to S18 transcripts in RT-PCR. The activity of inflammation was determined by histological evaluation of the peroperative colonic samples graded in three subgroups (mild, moderate and severe).

CFTR. In inflamed colonic mucosa, the CFTR mRNA expression was increased 2.5-fold compared to non-inflamed samples ($p=0.0002$, Mann-Whitney U-test), when all subgroups were analysed together. Characterization of the three subgroups of inflammation demonstrated upregulated

expression of the CFTR mRNA in all stages of inflamed tissues: about 5-fold in mildly inflamed samples ($p<0.001$), about 2-fold in moderate and ~1.5-fold in severely inflamed colon mucosa when compared to normal.

To assess putative alterations in the CFTR protein expression in response to inflammation, a set of inflammatory samples were studied using immunohistochemistry with a monoclonal CFTR antibody and compared to the normal control. The CFTR-specific immunoreactivity was detected only at the apical edge of the cryptal epithelial cells corresponding well with the functional CFTR protein at the apical membrane. All ulcerative colitis samples demonstrated the expression of the CFTR protein similar to the normal colon epithelium. Interestingly, some inflammatory samples demonstrated also cytoplasmic expression of the CFTR protein that was not detected in normal control samples.

SLC26A3. In contrast to CFTR, the expression levels of the SLC26A3 mRNA was not different between normal and inflamed mucosa, when all subgroups were analysed together ($p=0.11$) In different subgroups, an increased expression of SLC26A3 in mild inflammation (~1.5-fold) was followed by reduction in moderate (~2-fold) and severe inflammation (~3-fold). Expression of SLC26A3 was significantly changed ($p<0.05$) only in the severely inflamed tissue when compared to normal.

To study whether the levels of the SLC26A3 mRNA correlate with the protein expression levels, Western immunoblotting was performed using differently inflamed colon tissues. Antibody against the N-terminal part of the SLC26A3 protein detected a specific band of 85 kDa in all samples, and antibody against the C-terminal tail of the SLC26A3 peptide detected two protein bands at about 85 and 75 kDa. The intensities of the bands in mildly and severely inflamed samples appeared identical, suggesting that the expression of SLC26A3 remains unchanged even in severely inflamed colon tissue. These results were statistically verified by measuring a total of 30 immunoreactive bands from 10 patients by densitometric scanning of the autoradiographs. There was no significant difference ($p=0.8$) in mean protein expression in normal versus inflamed tissues. Characterization of the subgroups demonstrated also that SLC26A3 expression remains unchanged in all stages of inflamed tissues. However, the amino terminal part of the SLC26A3 protein might undergo posttranslational cleavage, because the N-terminal antibody detected only the larger 85 kDa band corresponding with the predicted molecular weight of the SLC26A3 protein. This observation is supported by the previous studies (Byeon et al. 1996).

SLC9A3. Similar to SLC26A3, the expression level of the SLC9A3 mRNA remained unchanged between normal and inflamed mucosa, when all subgroups were analysed together ($p=0.5$). In different subgroup, the initial upregulation in both mild (~3-fold) and moderate (~1.5-fold) was followed in severe inflammation by return to the same level as in non-inflamed samples. Statistically significant difference was seen only between mild and severe groups. These results are consistent with the previous studies demonstrating unchanged protein expression of SLC9A3 in an UC patient (Yang et al. 1998).

6. DISCUSSION

6.1. The SLC26 family is complemented with several new homologous members

The systematic characterization of gene families using genome sequences provides a rich source for expanding our physiological understanding of body functions. A second distinct family of anion exchangers, in addition to the classical SLC4 family, has recently been delineated. Particular interest in the SLC26 family is stimulated by the fact that the first three human genes, SLC26A2, SLC26A3 and SLC26A4, have been recognized as the disease genes mutated in diastrophic dysplasia, congenital chloride diarrhoea and Pendred syndrome, respectively. A growing interest is further feeded by the existence of new tissue-specific members, which might associate with diseases as well (Everett and Green 1999; Kere et al. 1999). By a genome-driven approach, we have identified seven novel loci with distinct tissue expression patterns. Currently, the expanded human SLC26 family includes at least eleven tissue-specific members, termed SLC26A1-A11 (Table 5).

We cloned SLC26A1, which likely represents the human ortholog of the rat and murine slc26a1 genes (sat-1) because of its high homology and expression pattern. Its transport function has not been determined yet, but it likely resembles that of rat and murine slc26a1 (Bissig et al. 1994). SLC26A2 to A4 have been shown to transport with different specificities the chloride, sulfate, iodide, bicarbonate, oxalate and hydroxyl anions, and they play central roles in the etiology of phenotypically very different recessive diseases (Hästbacka et al. 1994; Silberg et al. 1995; Satoh et al. 1998; Moseley et al. 1999; Scott et al. 1999; Scott and Karniski 2000).

During this study, the SLC26A5 gene (Pres), encoding 744-amino-acid protein, Prestin, was also cloned from gerbil and rat (Zheng et al. 2000; Ludwig et al. 2001). The human counterpart resides in chromosome 7, but remains to be cloned (Zheng et al. 2000). SLC26A5 acts as a new type of molecular motor in cochlea. It mediates changes in outer hair cell length in response to membrane potential variations, being responsible for sound amplification in the mammalian hearing organ. SLC26A5 uses monovalent anions as an extrinsic voltage sensor. After binding, these anions are translocated across the membrane in response to changes in the transmembrane voltage. This translocation triggers conformational changes in the protein that ultimately alters its surface area in the plane of the plasma membrane. Interestingly, SLC26A5 functions as an incomplete transporter, since it swings anions across the cell membrane but does not allow them to dissociate and escape to the extracellular space. Furthermore, SLC26A5 functions at microsecond rates, which is much faster than any other cellular motor protein (Oliver et al. 2001).

In this study, in addition to SLC26A1, we have cloned and functionally characterized four novel anion exchangers in chromosomes 3, 8, 6 and 1, named SLC26A6-A9, respectively. All four proteins showed distinct tissue-specific expression patterns, and functional expression demonstrated chloride, sulfate or oxalate transport activities. The chromosomal loci and initial tissue distribution of SLC26A10 and A11 are known but the genes remain poorly characterized so far. The characterization of these novel members may provide clinically important new insights to anion transport physiology in different parts of body.

Table 5. The SLC26 family of human anion exchangers.

Gene symbol	Chromosome location	Major sites of expression	Diseases	Sequence accession
SLC26A1 (SAT1)	4p16	Liver, pancreas, brain, kidney	None	AF297659
SLC26A2 (DTDST)	5q32	Ubiquitous	DTD, OMIM 222600 ACGIB, OMIM 600972 AOG2, OMIM 256050	U14528
SLC26A3 (CLD, DRA)	7q22-q31.1	Ileum, colon, seminal vesicle, eccrine sweat gland	Congenital chloride diarrhoea, OMIM 214700	L02785
SLC26A4 (PDS)	7q22-q31.1	Thyroid, kidney, cochlea	Pendred syndrome, OMIM 274600	AF030880
SLC26A5 (Prestin)	7	Outer hair cells of the cochlea	Not known	AC005064, (AF230376 for gerbil)
SLC26A6	3p21	Kidney, pancreas	Not known	AF279265
SLC26A7	8q21	Kidney	Not known	AF331521
SLC26A8 (Tat1)	6p21	Testis	Not known	AF331522
SLC26A9	1q32	Lung	Not known	AF331525
SLC26A10	12q14	Brain, other?	Not known	AF331523
SLC26A11	17q25	Ubiquitous	Not known	AF331524

The SLC26A6-A9 proteins are highly homologous to the previously known members, and comparison of the protein sequences revealed at least two common conserved domains within the family: sulfate transporter family and STAS domains (Table 4). The functional significance of these domains is still unclear. The STAS domain has been found from bacterial antisigma-factor antagonists (ASA) typified by *Bacillus subtilis* SPOIIAA and it has been found to express nucleotide binding activity (Najafi et al. 1996; Kovacs et al. 1998). Thus, the identification of the putative NTP-binding domain in the anion exchangers suggests that their function could be regulated by intracellular concentrations of GTP and/or ATP (Aravind and Koonin 2000). The critical role of the STAS domain is further supported by a number of diseases causing mutations

mapped to this region in the SLC26A2-SLC26A4 genes (Hästbacka et al. 1994; Superti-Furga et al. 1996; Everett et al. 1997; Höglund et al. 1998).

Sequence analysis revealed also that many SLC26 family members have a consensus PDZ-binding domain at their C-terminus (Table 4). The PDZ domain proteins play essential roles in maintaining the cell polarity and function by distributing receptors, transporters, ion channels and lipids asymmetrically between the apical and basolateral membranes (Fanning and Anderson 1999). Thus, the consensus PDZ motif found from the family members suggests that they could be regulated by PDZ-mediated interactions and supports the general concept of microdomain organization for ion transport.

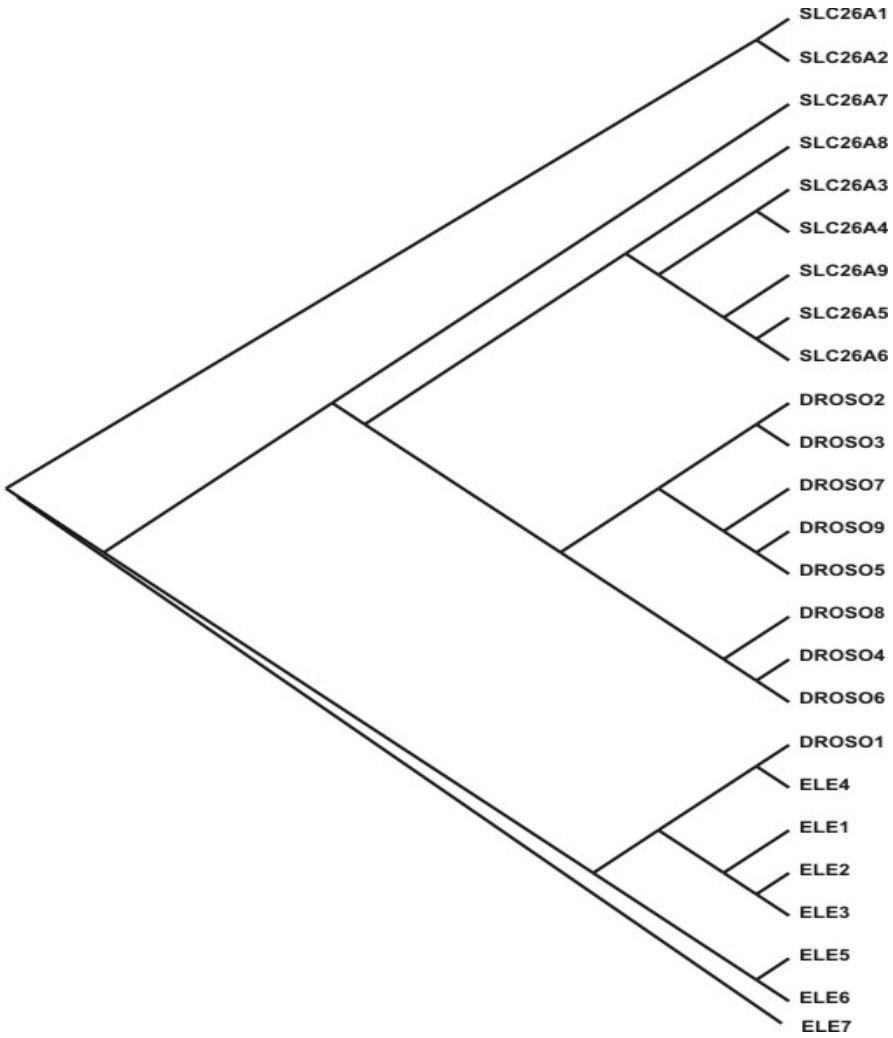

Figure 9. A phylogenetic analysis of SLC26 family in human, D melanogaster and C. elegans.

Phylogenetic analysis of the human, *D. melanogaster* and *C. elegans* family members illustrates the relationship of the exchangers with each other (Fig. 9). The SLC26 family members are mainly divided to three separate groups according to species. However, the human SLC26A1 and SLC26A2 proteins separate to their own branch. Structurally these two proteins resemble each other most, and their genomic structures are also very similar with three to four exons, which differs from the other human members consisting of about 20 exons. Diverging exon structure of SLC26A1 and A2 suggests that they may have lost most of their introns during evolution and resulted from a duplication event, although they have different chromosomal locations at 5q32 and 4p16, respectively. A similar but more recent duplication event has been suggested for the SLC26A3 and A4 genes, which reside within 40 kb of each other in chromosome 7 (Everett et al. 1997).

6.2. The novel SLC26 family members may have important tissue-specific functions

A striking feature of SLC26 family is the tissue-specificity of its members. The distribution of SLC26A3 and SLC26A4 is specific for the affected organs in congenital chloride diarrhoea and Pendred syndrome, respectively (Höglund et al. 1996; Everett et al. 1997). Functionally, the SLC26A3 protein is a major Cl^-/HCO_3^- exchanger in the gut and transports at least sulfate, oxalate and hydroxyl as well (Silberg et al. 1995; Melvin et al. 1999; Moseley et al. 1999), whereas SLC26A4 transports at least iodide, chloride and formate, but not sulfate (Scott and Karniski 2000). SLC26A2 expression is not restricted to the affected cartilage, but it can be found from many other tissues as well (Hästbacka et al. 1994; Haila et al. 2001). SLC26A5 is expressed only in the outer hair cells of the cochlea (Zheng et al. 2000). SLC26A6 is widely expressed, but at highest levels in kidney and pancreas. Consistently, the SLC26A7-A9 genes showed highly restricted expression patterns in kidney, testis and lung, respectively. Thus the tissue-specific expression likely points to important roles for the new members in these organs.

Several lines of evidence suggest SLC26A6 as a good candidate for a pancreatic ductal anion exchanger with a major physiological function. The primary function of the apical epithelium of the pancreatic duct is to secrete HCO_3^- anions (Gray et al. 1989). In pancreatic duct secretions from cystic fibrosis (CF) patients the HCO_3^- concentration is markedly reduced (Gaskin et al. 1982), and intraductal pH is sufficiently altered to precipitate proteins secreted from acinar cells (Freedman et al. 1998). In the currently accepted model of HCO_3^- secretion, the defect in secretion lies at the apical membrane of the ductal cells, where decreased secretion of Cl^- via CFTR inhibits the apical Cl^-/HCO_3^- exchanger. Recent studies suggest that the stimulation of CFTR selectively results in

activation of luminal Cl^-/HCO_3^- exchange activity in pancreatic ducts of mice and probably in other CFTR expressing cells (Lee et al. 1999). Furthermore, Cl^-/HCO_3^- exchange activity has been found in the basolateral and luminal membranes of mouse pancreatic ducts (Zhao et al. 1994; Zhao et al. 1995), but the protein responsible for luminal Cl^-/HCO_3^- activity has not been identified yet.

SLC26A6 protein was located to the apical and basolateral surfaces of tubular walls of human kidney and the apical surfaces of ductal pancreas and all SLC26A6 isoforms were expressed in a pancreatic ductal cell line, Capan-1. The high structural homology of SLC26A6 to known family members (SLC26A1-A5), which all transport Cl^- and HCO_3^- anions and demonstration of chloride, sulfate and oxalate transport activities further support the idea of SLC26A6 as a novel Cl^-/HCO_3^- exchanger. Finally, a recent study demonstrated that SLC26A6 indeed functions as a DIDS-sensitive Cl^-/HCO_3^- exchanger when expressed in *Xenopus laevis* oocytes (Wang et al. 2002).

The expression pattern and substrate specificity of the mouse slc26a6 proposes that it mediate several modes of anion exchange in a number of tissues, supporting our observations. Recently, a putative mouse orthologue of human SLC26A6, CFEX, (chloride-formate exchanger), was localized to the brush border membrane of renal proximal tubule cells and was demonstrated to mediate Cl^- -formate exchange when expressed in Xenopus oocytes (Knauf et al. 2001). Further examinations of different anions demonstrated that mouse slc26a6 has affinity for oxalate, sulfate and bicarbonate in addition to chloride and formate (Jiang et al. 2002). Mouse Slc26a6 can also function in multiple exchange modes involving pairs of these different anions, sharing the ability to mediate Cl^- -base exchange with slc26a3 and slc26a4 (Melvin et al. 1999; Soleimani et al. 2001; Jiang et al. 2002; Xie et al. 2002). Interestingly, the mouse slc26a6-mediated Cl^-/HCO_3^- and Cl^- /oxalate exchange elicits simultaneous membrane hyperpolarization, suggesting that it is an electrogenic transporter (Jiang et al. 2002; Xie et al. 2002).

Our results support also the general concept of microdomain organization for ion transport, and suggest a mechanism for CFTR-mediated SLC26A6 upregulation in pancreatic duct cells (Fig. 12). Recently, CFTR has been found to be necessary for the cAMP inhibition of HCO_3^- salvage, which is mediated by SLC9A3 and a yet unidentified Na^+ -dependent system. This regulatory interaction is mediated by binding of CFTR to PDZ1 and NHE3 to PDZ2 of NHERF (also known as EBP50) (Ahn et al. 2001). Therefore, our results, showing the interaction of SLC26A6 with PDZ1-2 domains of NHERF and E3KARP, suggest a similar regulatory mechanism for CFTR-directed luminal HCO_3^- secretion involving a CFTR-SLC26A6-NHERF complex. Upon pancreatic cell

stimulation, a complex of CFTR, NHERF (or E3KARP) and SLC26A6 might be assembled (or a pre-existing complex might be activated or modified) to stimulate HCO_3^- secretion, while the CFTR-NHERF-NHE3 complex inhibits HCO_3^- salvage (Fig. 10). This model is further supported by observations that CFTR induces the expression of SLC9A3, SLC26A3 and SLC26A6 (Ahn et al. 2001; Greeley et al. 2001). However, this model requires refinements and the exact composition of a putative multiprotein complex of NHERF or E3KARP, SLC26A6 and possibly CFTR or other proteins remains to be determined.

With regard to SLC26A6 and SLC26A6c (which carry the PDZ interaction motif) and SLC26A6d (which does not have the PDZ interaction motif) further studies will be needed to address the functional significance of the PDZ domain interaction motif for apical plasma membrane targeting and modulation of transport activity in polarised cells. PDZ interaction appears to be important, but not sufficient for targeting of CFTR to or retention of CFTR in the apical membrane in polarized cells (Short et al. 1998; Moyer et al. 2000). Interestingly, it has been observed that the binding of PDZ domain proteins to CFTR has a direct potentiating effect on channel activity (Wang et al. 2000; Raghuram et al. 2001). However, despite a number of studies the functional role of the PDZ interaction motif of CFTR remains somewhat unclear.

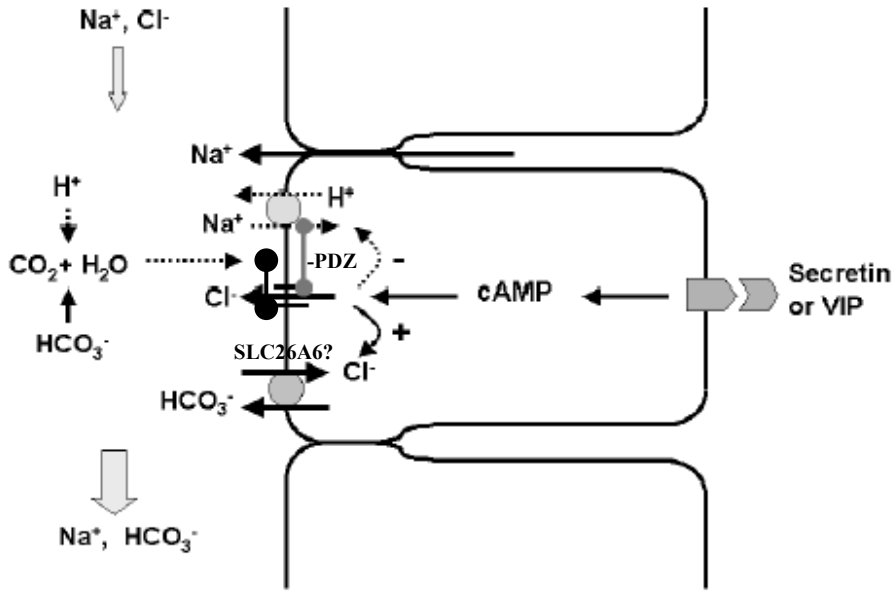

Figure 10. SLC26A6 may function as CFTR-directed Cl^-/HCO_3^- exchanger in the luminal side of the pancreatic duct. CFTR-mediated regulation of SLC26A6 may be controlled through PDZ mediated protein-protein interactions (modified from Ahn et al. 2001).

SLC26A6 and A7 are likely to play roles in the maintenance of the electrolyte and acid-base homeostasis in human kidney. SLC26A6 was localized to proximal and distal tubules and SLC26A7 specifically to the distal tubules, suggesting their cell type-specific anion transport functions in these nephron segments. The kidney is the major organ responsible for maintaining electrolyte balance and acid-base homeostasis in mammals. This is accomplished mainly by absorption of NaCl and secretion of acid or base equivalents in different segments of the nephron (Alpern et al. 1991). Functional studies using perfused tubules and isolated brush border vesicles have led to the concept that a major fraction of Cl^- entry across the apical membrane of proximal tubule cells occurs via Cl^-/formate and Cl^-/oxalate exchanges (Aronson and Giebisch 1997). However, although functional studies have characterized many of these transporters, their molecular identities have remained largely unknown, and still unknown acid-base transporters are likely to exist (Wang et al. 1993; Tsuruoka and Schwartz 1999; Soleimani et al. 2001).

Furthermore, defects in some of the renal transport systems have been associated with different diseases such as autosomal dominant and recessive distal renal tubular acidosis. The dominant form has been associated with mutations in the basolateral SLC4A1 (Bruce et al. 1997), and in addition to two known loci, ATB6B1 and rdRTA2, yet further unknown loci are suggested (Karet et al. 1998; Karet 1999; Karet et al. 1999). Therefore, further studies regarding the function and expression of the SLC26A6 and -A7 proteins in different renal pathophysological states are clearly warranted.

SLC26A7 expression was observed also in HEV cells (Vincourt et al. 2002). HEVEC are known to incorporate large amounts of inorganic sulfate, and sulfate residues are essential for the recognition of HEVEC sialomucins by L-selectin, a cell adhesion molecule that mediates the initial interaction of lymphocytes with HEVEC (Girard and Springer 1995; Vincourt et al. 2002). Previously, SLC26A2 and a novel sodium-dependent sulfate transporter, SLC13A4, have been proposed to mediate the sulfate uptake into human HEVEC (Girard et al. 1999). Our demonstration of the sulfate transport function of SLC26A7 suggests a possible cooperative role in sulfation of HEV sialomucins and regulation of lymphocyte migration in organized lymphoid tissues at sites of chronic inflammation.

The testis is composed of loops of seminiferous tubules lined with epithelium containing Sertoli cells and spermatogenic cells at various stages of differentiation. The seminiferous tubule fluid establishes the microenvironment for spermatogenesis and transports spermatozoa from the tubules

to the head of the epididymis. The specific composition of the fluid produced by the Sertoli cells may play a vital role in germ cell development (Tuck et al. 1970). The germ cell-specific expression of SLC26A8 suggests a functional role in these cells. The extensive morphological changes observed during spermatogenesis suggest that the regulation of ion transport is a critical component of this differentiation process.

SLC26A8 expression was limited to the meiotic phase of the development, since strong staining was seen only in the spermatocytes and developing spermatids. Because SLC26A8 demonstrated chloride, sulfate and oxalate transport activities, our results suggest its function as a novel male germ cell-specific anion exchanger, which may be directly required for the development and maturation of these lineages. Specifically, sulfate transport may play an important role for germ cell development, as observed previously for the development of the cartilage in DTD patients (Hästbacka et al. 1994). Moreover, SLC26A8 has been linked to RhoGTPase signaling, which plays an important role in the development and function of male germ cells (Toure et al. 2001). Finally, SLC26A8 function may also affect the composition of the seminal fluid and participate in the regulation of the Sertoli cells. Of importance, failure of spermatogenesis has recently been associated with impaired transport function in mouse deficient of the Na^+-K^+-$2Cl^-$ cotransporter (Pace et al. 2000). Thus, defective function of SLC26A8 might also result in impaired spermatogenesis, making the gene a plausible candidate for male infertility.

Functional characterization of SLC26A9 as a DIDS-sensitive anion exchanger may cast more light into the anion transport physiology of human lung. Airway epithelia is involved in both fluid and electrolyte absorption and secretion. Various mechanisms involving Na^+ and Cl^- transport have been identified, including electrogenic Na^+ and Cl^- channels, and electroneutral Na^+/H^+ exchange and Cl^-/HCO_3^- exchange (Welsh 1987; Tse et al. 1993; Voilley et al. 1993; Alper 1994; Yun et al. 1995). SLC4A2 and A3 anion exchangers have been found in human airways (Dudeja et al. 1999; Loffing et al. 2000). Although detailed functional roles of SLC26A9 remain to be defined, its expression in the bronchial and alveolar cells of distal lung suggest a role in the maintenance of intracellular pH of the epithelial airway cells and possibly also the pH of airway surface liquid. The distal airways including medium and small terminal bronchioles cover >80% of the total mucus membrane surface area of the conducting airways, and play a pivotal role in formation and regulation of airway secretion (Boucher 1994).

The regulation of ion transport and the composition of ASL are important parts of lung defence mechanisms and may contribute to different airway diseases (Noone et al. 1994). Recent studies suggest that the composition of ASL is regulated by active ion transport systems (Tarran et al. 2001). In cystic fibrosis, defective secretion of chloride and hence excessive ASL absorption results in a characteristic syndrome with retained secretions, bacterial infection, and lung destruction (Sferra and Collins 1993). Conversely, pseudohypoaldosteronism patients, who exhibit loss-of-function mutations in ENaC, have an excess of ASL due to reduced Na^+ absorption. This leads to drastic acceleration of the mucus transport (Kerem et al. 1999). SLC26A9 may participate in the regulation of volume and composition of the ASL as well as in the sulfation of the mucosal components, and its defects in transport function may associate with diseases of the human respiratory system.

6.3. Altered expression of transporters may contribute to diarrhoea in intestinal inflammation

The main function of the human colon is to absorb about 90% of over 2 litres of Na^+- and Cl^--rich fluid, which passes daily through the small intestine (Debongnie and Phillips 1978). In normal colonic mucosa, the rates of salt and water absorption are directly related, and the main factor determining the extent of water movement out of the colonic lumen is the rate of Na^+ absorption (Binder and Sandle 1994). The human colon possesses an array of Na^+ absorptive mechanisms that are not distributed uniformly throughout its length. Much of the Na^+ absorption is mediated by electroneutral pH-coupled Na^+/H^+ and Cl^-/HCO^-_3 exchangers (Knickelbein et al. 1985). However, there are also apical sodium channels, which are involved in electrogenic sodium transport (Canessa et al. 1994). Instead, CFTR seems to be the only pathway for Cl^- exit in the colonic epithelium (Mall et al. 1998).

CFTR have been shown to play a central role in coordinating electrolyte transport in various tissues including the intestines, pancreas, lungs, sweat glands, and kidneys. CFTR is a member of the ATP-binding cassette (ABC) membrane transporter superfamily (Riordan et al. 1989). It contains 1480 amino acids and consists of two homologous halves with six membrane-spanning segments and a nucleotide-binding domain. The two halves of CFTR are linked by a cytoplasmic regulatory domain (R-domain) that contains a number of consensus phosphorylation sites (Riordan et al. 1989). CFTR forms a cAMP- and Ca^{2+}-activated Cl^- channel, which requires ATP hydrolysis for the channel to open and close (Gadsby and Nairn 1999).

Defects in CFTR expression or functions cause cystic fibrosis (CF, OMIM #219700) and a form of male sterility due to congenital bilateral absence of the vas deferens (De Braekeleer and Ferec 1996). In contrast, overstimulation of Cl^- secretion, e.g. by bacterial enterotoxins, is associated with secretory diarrhoea (Vaandrager et al. 1997). CF presents defective fluid and electrolyte secretion in the respiratory, pancreatic, intestinal and genitourinary systems (Riordan et al. 1989; Pilewski and Frizzell 1999). The intestinal and lungal phenotypes of CF result from luminal obstruction by thick mucoid secretions (Eggermont and De Boeck 1991). In pancreatic duct secretions from CF patients the HCO_3^- concentration is markedly reduced (Gaskin et al. 1982), and intraductal pH is sufficiently altered to precipitate proteins secreted from acinar cells (Freedman et al. 1998). The defect in secretion lies at the apical membrane of the ductal cells, where defective CFTR inhibits the apical Cl^-/HCO_3^- exchanger.

The function of the colonic epithelium includes the regulation of water and ion transport as well as the formation of an effective barrier against the invasion of noxious agents and antigens. Both functions seem to be impaired in ulcerative colitis, leading to intestinal inflammation and diarrhoea in the course of disease. The pathophysiological mechanisms of diarrhoea in UC are not completely known, although various investigations have been made concerning the contributory role of the activation of secretion (Rampton and Laden 1984), the reduction of ion absorption (Harris and Shields 1970; Rask-Madsen 1973), and a defect in epithelial barrier function (Sandle et al. 1990).

To understand better the pathophysiology of diarrhoea in UC, we examined the effect of intestinal inflammation on the expression of three intestinal electrolyte transporter genes, suggested to account for the major colonic NaCl and fluid movements: SLC26A3, SLC9A3 and CFTR. Further interest in these genes is stimulated by the fact that their functional defects have been associated with diarrhoeal diseases such as congenital chloride diarrhoea and sodium diarrhoea; and a distinct phenotype with lack of secretory response and meconium ileus seen in cystic fibrosis (Guerrant et al. 1990; Schultheis et al. 1998; Kere et al. 1999). In addition, the CFTR-induced upregulation of the SLC26A3 and SLC9A3 mRNA expression in heterologous systems supports the idea that the three proteins work in concert with each other (Wheat et al. 2000; Ahn et al. 2001; Greeley et al. 2001). Thus, defects in any of these proteins may affect the expression and function of the others as observed with the SLC9A3 knockout mice demonstrating the upregulation of the SLC26A3 gene (Melvin et al. 1999).

Although CFTR has a crucial role in the regulation of the intestinal electrolyte homeostasis, not much is known about its expression or function in inflamed colon mucosa. In general, inflammatory stimulation results both in an increased epithelial permeability and altered patterns of proliferation and differentiation of intestinal epithelial cells (Madara and Stafford 1989; Ziambaras et al. 1996). Elevated CFTR expression and enhanced cAMP-dependent Cl^- secretion have been demonstrated in hyperproliferated mouse intestine (Umar et al. 2000). In addition to Cl^- secretion, CFTR has been shown to regulate the function and properties of Na^+ and K^+ channels, the Cl^-/HCO^-_3 exchanger, Na^+/H^+ exchanger, mucous secretion and, secondarily, water permeability (Kunzelmann et al. 2000). Thus, the upregulation of CFTR, as shown in our series of inflamed tissues, might result in both increased secretion of Cl^- and decreased absorption of sodium of the mucosa, and ultimately contribute to diarrhoea.

Previous studies have produced ambiguous results demonstrating decreased or unchanged CFTR expression in intestinal epithelium (Madara and Stafford 1989; Besancon et al. 1994; Colgan et al. 1994). In our study, the expression of the CFTR protein was detected both in healthy and inflamed colon mucosa by immunohistochemistry, although the overall expression level of CFTR in colon is not abundant. Interestingly, some inflamed colon tissue samples demonstrated cytoplasmic expression of CFTR that could not be detected in normal control. It corresponds likely to a newly synthesized or retarded form of the CFTR protein in intracellular compartments, and might suggest that the translation of the CFTR protein is enhanced in the inflamed colon mucosa.

Both the activation of ion secretion and the reduction of ion absorption have been suggested as the pathomechanisms in diarrhoea in UC. The inflamed colonic mucosa contains increased levels of different inflammatory mediators, which are capable of triggering Cl^- secretion. The secretion of chloride and accompanying secretion of water are thought to be important transport abnormalities contributing to the pathogenesis of diarrhoea in UC (Wardle et al. 1993; Wardle and Turnberg 1994). However, there is also a limited number of human studies as well as animal models of colitis supporting the idea that diarrhoea in IBD is likely due to anti-absorptive rather than pro-secretory effects (Greig and Sandle 2000). The high lumen-negative transmucosal electrical potential difference present in normal colon is decreased in UC patients, suggesting loss of electrogenic Na^+ absorption rather than enhanced Cl^- secretion (Sandle et al. 1986; Sandle et al. 1990). In addition, malabsorption of Na^+ and Cl^- has also been associated with inflamed colonic mucosa (Harris and Shields 1970; Edmonds and Pilcher 1973; Hawker et al. 1980; Bell et al. 1995; Sundaram and West 1997) as well as a notably diminished activity of basolateral membrane Na^+-

K^+-ATPase, which is a prerequisite for apical Cl^- secretion (Sandle et al. 1990). These studies indicate that impaired water absorption secondary to impaired Na^+ and Cl^- absorption rather than Cl^- secretion is a major pathogenic factor in the diarrhoea of acute colitis. Therefore, further studies are still required to correlate the putative functional changes in inflamed human colon with the elevated level of CFTR and its putative intracellular redistribution found in this study.

This study supports our earlier findings demonstrating similar expression of the SLC26A3 mRNA in inflammatory bowel disease and ischemic colitis when compared with normal colon epithelium (Haila et al. 2000). In this study, there were no significant changes in the expression of the SLC26A3 and SLC9A3 genes in ulcerative colitis compared with non-inflamed tissues, when all subgroups were analysed together. However, there is variation in the individual levels of expression of the genes, especially with SLC9A3, which may reflect alterations in region-specific expression of the genes, because the tissue samples were collected at different locations along the colon. Furthermore, all the patients were treated with glucocorticosteroids known to alleviate intestinal inflammation and modulate the expression of some genes involved in transport processes; e.g., SLC9A3 (Ambuhl et al. 1999; Kiela et al. 2000).

Characterization of the effect of disease activity on epithelial gene expression revealed slight changes for SLC26A3 and SLC9A3. An initial increase in mild disease was followed by a decrease in moderate and severe UC in the case of SLC26A3. SLC9A3 expression increased more than that of SLC26A3 in mild UC, but remained unchanged in severe UC. Speculatively, the slight but insignificant increase in the expression of SLC26A3 and SLC9A3 in mildly inflamed intestine could also reflect an increased activation of CFTR as shown previously in other expression systems. There are several reasons that may be responsible for the reduction of the SLC26A3 mRNA seen in severe UC. It may be secondary, due to a loss of epithelial cells by erosions and ulceration, or due to an expansion of an undifferentiated crypt cell compartment. However, no changes in the expression of the SLC26A3 protein was noted even in severe UC. In our earlier study, we observed the expansion of the SLC26A3 expression deeper in the crypts to the cells of the proliferative cell compartment in a set of inflammatory colon samples (Haila et al. 2000). Similarly, SLC9A3 staining has been suggested to remain unchanged in a colon tissue specimen of an UC patient (Yang et al. 1998). It is likely that the protein translation machinery in inflamed intestinal cells can compensate small fluctuation in the mRNA level.

A rabbit model of chronic ileal inflammation has demonstrated that the inhibition of coupled NaCl absorption by the villus cells occurs as a result of diminished Cl^-/HCO^-_3 but not Na^+/H^+ exchange activity (Sundaram and West 1997). The mechanism has been shown to be a decrease in affinity for Cl^- rather than an altered number of transporters. Since there were no significant changes in the levels of SLC26A3 and SLC9A3 transporters even in severe colitis, it is tempting to speculate that a similar model of inhibition of NaCl absorption might exist also in human colon. However, several different studies using different conditions or models have produced ambiguous results regarding the regulation of the expression of different apical and basolateral transporters (Madara and Stafford 1989; Sandle et al. 1990; Besancon et al. 1994; Colgan et al. 1994; Yang et al. 1998). Moreover, significant differences may occur in different models and there may be differences even between UC and other types of intestinal inflammation.

In summary, intestinal inflammation modulates the expression of these major mediators of intestinal salt transport and may contribute to diarrhoea in ulcerative colitis both by increasing transepithelial Cl^- secretion and by inhibiting the epithelial NaCl absorption, but a simple clear picture of a single mechanism has not emerged.

CONCLUSIONS AND FUTURE PROSPECTS

As an example of the fruits of genome projects, this study describes the expansion of the SLC26 family using a genome-driven approach. Subsequently, seven novel loci (designated SLC26A1, A6-A11) with distinct tissue expression patterns were identified and mapped, demonstrating the power of the genomic approach in the identification of new genes. The preliminary characterization of the structure, expression and function of the SLC26A6-A9 genes suggest that the novel members are likely to participate in important physiological functions involving anion transport in different organs and might associate with disorders.

It is obvious that much work has to be done to define the cellular and molecular mechanisms underlying the function, expression and regulation of the new family members. However, mapping, cloning and functional characterization of the new genes provide now a rich source for additional physiological studies. In addition, determination of the genomic structures enables the screening of possible mutations in diseases. Characterization of new genes facilitates also the cloning of the orthologs from other species. More specifically, targeted disruption of the mouse orthologs would further help to understand the physiological roles of the new SLC26 family members in respective tissues.

Several lines of evidence support the idea of SLC26A6 as a good candidate for a yet unknown, CFTR-regulated protein responsible for the luminal Cl^-/HCO^-_3 exchange in pancreas. First, SLC26A6 is expressed in the apical surface of pancreatic ducts. Second, SLC26A6 and its two alternatively spliced variants demonstrated chloride transport activity. Third, the C-terminus of SLC26A6 interacted with the PDZ domains of the E3KARP and NHERF proteins in vitro. Recently, CFTR has been found to be necessary for the cAMP inhibition of HCO_3^- salvage mediated partly by SLC9A3 and this regulatory interaction is mediated by binding of CFTR to PDZ1 and NHE3 to PDZ2 of NHERF (Ahn et al. 2001). Thus, our results suggest a mechanism for CFTR-mediated SLC26A6 upregulation in pancreatic duct cells, and support the general concept of microdomain organization for ion transport.

Although further studies are needed to refine this coarse model, our results suggest a clinically important role for SLC26A6 in pancreatic HCO_3^- metabolism. In pancreatic duct secretions from cystic fibrosis patients the HCO_3^- concentration is markedly reduced and intraductal pH is sufficiently altered to precipitate proteins secreted from acinar cells. The defect in HCO_3^- secretion

lies at the apical membrane of the ductal cells, where decreased secretion of Cl- via CFTR inhibits the apical Cl- /HCO3- exchanger.

The first three human genes, SLC26A2-A4, associate with phenotypically different disorders, involving cartilage growth, gut absorption, and hearing, respectively. Thus, the three closely related but highly tissue-specific human anion transporters play central roles in the etiology of phenotypically very different recessive diseases. Similarly, the tissue-specific expression of SLC26A7-A9 in kidney, testis and lung, respectively, suggest important cell-specific anion transport functions. SLC26A7 localized to distal segments of nephrons, SLC26A8 to developing spermatocytes, and SLC26A9 to the lumenal side of the bronchiolar and alveolar epithelium of lung. Furthermore, all three proteins demonstrated chloride, sulfate and oxalate transport activity. Thus, SLC26A7 is likely to play a role in the maintenance of the electrolyte and acid-base homeostasis in human kidney. Defective function of SLC26A8 might result in impaired spermatogenesis, making the gene a possible candidate for male infertility. SLC26A9 may participate in the regulation of the airway surface liquid, which is an important part of the lung defence systems, and impaired function of SLC26A9 might associate with diseases of the human respiratory system. Therefore, further studies regarding the function and expression of the proteins in different pathophysological states are clearly warranted.

In this study, as an example of the physiological role of ion transporters in pathophysiological states, we investigated how intestinal inflammation affects the expression of three major transporters of intestinal salt transport, namely SLC26A3 (Cl^-/HCO_3^--exchanger), SLC9A3 (Na^+/H^+-exchanger) and CFTR (Cl^--channel), to understand the pathophysiology of diarrhoea in ulcerative colitis. All three transporters are thought to play in concert with each other, and their functional defects have been associated with diarrhoeal diseases. Our results indicate that intestinal inflammation modulates the expression of these major mediators of intestinal salt transport and may thus contribute to diarrhoea in ulcerative colitis both by increasing transepithelial Cl^- secretion and by inhibiting the epithelial NaCl absorption. However, a simple clear picture of a single mechanism has not emerged and additional functional experiments are needed to understand better the pathophysiology of diarrhoea in inflammatory bowel diseases.

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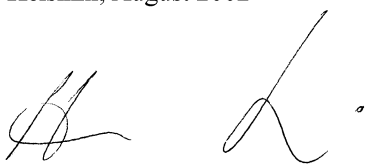

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